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STUDIES OF HEMAGGLUTININS IN HEREDITARY SPHEROCYTOSIS AND IN ACQUIRED HEMOLYTIC ANEMIA; THEIR RELATIONSHIP TO THE HYPERSPLENIC MECHANISM

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THE importance of an unsuspected immunologic phase in some hitherto obscure mechanisms of disease is gradually becoming apparent. Newer techniques are revealing significant but previously unrecognized antigen-antibody reactions, as for example the discovery of "incomplete antibodies" in certain of the hemolytic anemias.[1-27] The detection of these antibodies, in some instances, has clarified previously unexplained fundamental pathologic phenomena, in others a differential diagnostic tool has been added. The purpose of this paper is to present the results of a study of incomplete antibodies as revealed by these newer methods when applied to hereditary spherocytosis and to certain acquired hemolytic anemias, and to consider their relationship to the mechanism of hypersplenism.

The concept of incomplete antibodies is not really new, though means for demonstrating them have been developed and applied clinically only within recent years. In 1902, Eisenberg and Volk[28] described bacterial "agglutinoids" which probably are related to if not identical with today's "incomplete," "blocking" or "monovalent" type of antibodies. So-called "monovalent" antibodies were described by Heidelberger and Kendall[29] in 1935. In 1944, Race[1] recognized the "incomplete" antibody in Rh-negative mothers who had been sensitized by fetal Rh-positive erythrocytes. In the same year, Weiner[2] demonstrated his "blocking" antibody with a saline suspension of erythrocytes. Diamond and Abelson,[3] in 1945, utilized plasma in the development of a slide test, and later the same year Diamond and Denton[4] introduced the use of bovine albumin as the diluent. In 1945, Coombs, Mourant, and Race[5] prepared in the rabbit an antihuman globulin serum (developing serum or Coombs serum) which detected incomplete Rh agglutinins absorbed on red blood cells. A modified indirect technique was advocated for demonstrating incomplete antibodies in the serum.

Wheeler and associates[6, 7] very recently have standardized a still more sensitive test by using enzyme-treated red cells for detecting serum incomplete

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Rh antibodies. It had been observed by several investigators that Rh-positive red blood cells treated by viruses or enzymes are agglutinated in very high saline dilutions of blocking anti-Rh serum. Burnet, McCrea, and Stone[8] first noted this phenomenon with cells treated with virus and filtrates of *Vibrio cholerae*. Morton and Pickles[9] used crude and crystalline trypsin and noted similar characteristics. Quilligan[10] found that pepsin, papain, erypsin were also effective. Wheeler added chymotrypsin, bromelin (fresh pineapple juice), and Russell viper venom (Stypven). The standardized trypsin-treated red blood cell technique has proved to be a simple, reliable, and sensitive screening test in the detection of anti-Rh antibodies.

In addition to the studies in antibody hemagglutination, Rheins, Dodd, and Hudson[30] have shown that trypsin-treated groups O and A human erythrocytes are agglutinated to considerably higher titers with viable influenza virus as compared with trypsinized groups B and AB erythrocytes.

We have utilized in our studies of the potential immunologic aspects of the hypersplenic syndromes both the developing serum (Coombs) test which provides a method for detecting incomplete antibodies *on the red cell* and the trypsinized red blood cell technique which determines the presence of incomplete antibodies *in the serum*. A study characterizing such enzymic effects on erythrocytes will be reported elsewhere.

MATERIALS AND METHODS

I. *Developing Serum (Coombs) Test.*—

A. Preparation of Anti-Human Globulin: 1. Preparation of Alum-Precipitated Serum Protein. Five cubic centimeters of serum from normal group O blood are diluted with 16 c.c. distilled water in a 50 c.c. centrifuge tube and 18 c.c. of a 10 per cent solution of potassium aluminum sulfate (in distilled water) are added. The pH is adjusted to 6.5 with N/5 NaOH. After centrifugation at 1,500 r.p.m. for five minutes the supernatant fluid is removed and 40 c.c. of physiologic saline-sodium ethylmercurithiosalicylate* (0.01 per cent) are added. Repeat centrifugation and washing three times. After the third procedure, 20 c.c. of saline are added to the protein precipitate and the solution is ready for injection.

2. Rabbit Immunization. Ten cubic centimeters of alum-precipitated serum protein are injected into a 2 kilogram rabbit (5 c.c. into each thigh). Titers are determined weekly by the precipitin ring test. If a 1:10,000 titer is not obtained by the twenty-first day following the first injection, a second injection is given. Three weeks after the last injection if there is a 1:10,000 minimum titer, the animal is exsanguinated via the heart, using a 50 c.c. syringe and 18 gauge needle. Permit the blood to clot; remove serum and preserve by adding sodium ethylmercurithiosalicylate to obtain a final 1:10,000 dilution.

3. Absorption of Anti-Serum. The complement is inactivated by heating at 56° C. for thirty minutes. Absorption of the serum is accomplished with packed, washed (five times with normal saline) red cells. Approximately 4 c.c. of the serum are absorbed with 1 c.c. *each* of packed A_1, A_2, B, and O cells (totalling 4 c.c. cells) for ten minutes at 37° C. with frequent agitation. This procedure is repeated the second time for twenty minutes and the third time for thirty minutes.

A check of the serum after absorption is made in the following manner. A 2 per cent suspension of three times washed A_1, A_2, B, and O cells† (0.7 c.c.) is added to 0.1 c.c. of absorbed serum. Read for agglutination after incubation for thirty minutes at 37° C.

*This preservative (Merthiolate, Lilly) was added to all physiologic saline used in this procedure.

†A minimal of four different samples of each blood group used.

If agglutination occurs after the third absorption, the procedure is again repeated for one hour at room temperature. The serum used in the developing test must have all agglutinins for A_1, A_2, B, and O cells removed.

Absorbed antiserum titer is determined by one or both of two methods, the precipitin ring test (1:1,000 titer satisfactory) and the agglutination test as modified by Wagley and co-workers[31] (1:128 titer satisfactory).

B. Technique:

To serial saline dilutions of 0.1 c.c. developing serum add 0.07 c.c. of 2 per cent suspension of packed washed (three times in cold saline) test red blood cells. Agitate and incubate at 37° C. for thirty minutes. Centrifuge one minute at 1,800 r.p.m. Read for agglutination.

II. *The Trypsinized Red Blood Cell Test.—*

A. Preparation of Trypsinized Red Blood Cells:

One cubic centimeter of washed (three times in cold saline*), packed group O red blood cells are added to 1.5 c.c. of a 0.1 per cent trypsin†-saline solution. This suspension is incubated for thirty minutes at 37° C., with frequent agitation. After centrifugation, remove the supernatant and wash enzymized red cells three times with cold saline. Make a 2 per cent suspension in saline.‡ Suspension is checked for nonspecific agglutination with normal sera.

B. Technique:

Serial saline dilutions are made with 0.1 c.c. of patient's serum; 0.07 c.c. of the 2 per cent trypsinized red cell suspension is added. The mixture is agitated; let stand for one to two minutes; centrifuge for one minute at 1,800 r.p.m. Read for agglutination.

Normal sera are run as controls.

III. *Immunologic Studies of Splenic Blood.—*

A. Terminology of Splenic Blood Specimens:

We have drawn attention to the functional analogy which exists between the spleen and the lungs.[32] This suggested parallelism yields terms as well as concepts facilitating a better understanding of the normal and pathologic splenic mechanisms. The blood entering and leaving the spleen under ordinary basal conditions is comparable to the *tidal air* during normal respiration, and it may be appropriately designated as *splenic tidal blood*. Complete voluntary expiration (*reserve air*) followed by a deep inspiration (*complemental air*) measures the pulmonary vital capacity. Similarly, relaxation of the spleen as obtained with sodium pentobarbital,[33] followed by a maximum contraction induced by epinephrine, will reveal the *vital capacity of the spleen* as a reservoir for sequestered blood elements. Epinephrine stimulation without previously induced relaxation mobilizes the *splenic blood reserve*. The *residual air* represents the irreducible minimum retained within the pulmonary alveoli in the intact chest and is comparable to the sinusoidal-parenchymal *residual blood* obtained only by further manual manipulation of an epinephrine-contracted spleen after its surgical removal.

These specific terms, i.e. *splenic tidal blood*, *splenic reserve blood*, and *splenic residual blood*, are used to designate the contents of the various splenic compartments.

B. Technique of Obtaining Specimens:

These studies were made at the time of splenectomy advised for one of the hypersplenic syndromes. The blood specimens from splenic artery and vein were obtained using the following surgical technique (devised and carried out by R. M. Z.). A high left paramedian incision is made into the peritoneal cavity and *extreme care is taken not to disturb the spleen mechanically*, even when massively enlarged. The gastrosplenic ligament is divided along the greater curvature in the region of the fundus of the stomach, giving

*All saline used in preparation of the trypsinized red blood cells is physiologic and phosphate buffered to pH 7.28.

†Difco trypsin, 1:250 activity, used.

‡These test cells are satisfactory for forty-eight hours when stored at 4° C. It is preferable to make up fresh test cells three times weekly when using them daily. After forty-eight hours the cells begin to hemolyze and show progressively diminishing titers and avidity.

direct entrance to the lesser sac. The opening into the lesser sac is enlarged until the splenic artery can be easily visualized as it courses along the upper margin of the body of the pancreas. The peritoneum over the vessels is carefully incised about 8 or 10 cm. from the hilus of the spleen and the artery gently dissected from its bed for a short distance by the use of a long right-angle clamp. Two traction sutures of silk are placed beneath the artery.

Since there is a tendency for the splenic artery to contract following such manipulation in the younger age group with nonsclerotic vessels, direct aspiration of the artery was frequently carried out without previously isolating it from its bed. In many of the cases, traction sutures were likewise placed beneath the deeper situated splenic vein; however, this is unnecessary since bleeding from the puncture wounds can easily be controlled with small pledgets of Gelfoam.

After the field is prepared, 10 to 17 c.c. of blood are aspirated from the splenic vein, utilizing a number 20 needle with a 20 c.c. syringe. This specimen is representative of *splenic tidal blood*. Following this, a similar aspiration is made from the splenic artery. With the needle remaining in place in the artery, 0.3 to 1.0 c.c. of epinephrine (depending on the age and the cardiovascular status of the patient) is then slowly injected into the splenic artery. Following the completion of the injection, a small amount of arterial blood is aspirated into and reinjected from the syringe to complete the accurate injection of all the epinephrine directly into the splenic artery. When there is a significant rise in pulse and blood pressure associated with the visible contraction of the spleen (from two to five minutes), a second aspiration is made from the splenic vein (*splenic reserve blood*). Gelfoam (Upjohn) is immediately applied to each aspiration site as the needle is withdrawn. Difficulties with troublesome bleeding have not been encountered. Following this, the traction sutures beneath the splenic artery are tied, effecting a double ligation of this vessel. In the majority of the cases the splenic vein was not ligated at the time of aspiration, but continued to return blood from the spleen to the general circulation while the spleen was mobilized and the pedicle made ready for ligation. The splenic vein is preferably the last vessel ligated. After the removal of the spleen, a careful search is made for accessory spleens. A biopsy of the liver and the removal of a mesenteric lymph node are done routinely in all cases. Careful hemostasis with individual ligation of all blood vessels minimizes blood loss during the procedure. No unfavorable reactions or effects due to these procedures were observed in any patients in this series.

The aspirated specimens, as collected by the surgeon, and the *residual blood* specimen are handled by the hematologist, who ejects samples into cupped paraffin plates and the hematologic preparations for qualitative and quantitative study are made immediately in the operating room; these results are being reported in a separate publication.[32] The remainder of each specimen is saved for immunologic studies with the techniques described and these accumulated data are reported here (Fig. 6).

After ligation of the hilar vessels, the spleen is removed in the usual manner. The ligatures on the splenic side are immediately released and a specimen of the remaining *residual blood* is expressed from the splenic sinusoids and/or pulp by manual massage.

RESULTS

In applying the trypsinized red blood cell and the developing serum techniques, a total of 1,716 determinations has been made on blood samples from 465 persons: 185 were patients, each with some hematologic abnormality,* 133

*The diseases included hereditary spherocytosis, acquired hemolytic anemia, thalassemia, essential thrombocytopenic purpura, splenic neutropenia, splenic panhematopenia, sickle-cell anemia, Gaucher's disease, Banti's syndrome, Hodgkin's syndrome, erythroblastosis fetalis, incompatible transfusion reactions, multiple myeloma, aplastic anemia, toxic panmarrow hypoplasia, polycythemia rubra vera, pernicious anemia, macrocytic anemia of pregnancy, carcinoma of stomach, posthemorrhagic gastric ulcer, myelogenous leucemia, lymphatic leucemia, lymphosarcoma, monocytic leucemia, hepatic cirrhosis, infectious hepatitis, iron deficiency anemia, post acute hemorrhage anemia, cyclic neutropenia, rheumatic heart disease, myelofibrosis, bronchiectasis, and hyperthyroidism.

HEREDITARY SPHEROCYTOSIS

PERIPHERAL BLOOD TITERS WITH TRBC AND DEVELOPING SERUM (Coombs')

SUMMARY : TOTAL PATIENTS STUDIED = 25

POSITIVE TRBC : 6 | POSITIVE BOTH : 2

POSITIVE D.S. : 4 | NEGATIVE BOTH : 17

CASE	TITER: TRBC: + D.S.: + U	2	4	8	16	32	64	COMMENT RE: SPLENECTOMY	COMMENT GENERAL
1. D.B. ♀ Aged 19yrs.	+++ / ++	− / +	− / +	− / +	− / ±	− / −	− / −	POSITIVE BEFORE AND AFTER	Patient; severe hemolysis since birth
2. R.S. ♀ Aged 40 yrs.	− / ++	− / ++	− / +	− / −	− / −	− / −	− / −	NO	Mother; moderate hemolysis
3. E.B. ♀ Aged 17yrs.	− / ++	− / +	− / +	− / ±	− / −	− / −	− / −	NO	Sister; no hemolysis, increased fragilities.
4. J.G. ♂ Aged 17yrs.	+ / −	− / −	− / −	− / −	− / −	− / −	− / −	POSITIVE 4 MOS. AFTER	1st cousin; studies not done during hemolytic crisis precipitated by septicemia.
5. G.P. ♀ Aged 21yrs.	++++ / −	++++ / −	+++ / −	++ / −	+ / −	− / −	− / −	POSITIVE 10 YRS. AFTER	Splenectomy for acute crisis at 4 years. Remission for 4 years. re-exacerbation corrected by removal 5gm. accessory splenic tissue. Present remission continues in its 12th year.
6. H.H. ♀ Aged 62 yrs.	++ / ++	+ / +	− / ±	− / −	− / −	− / −	− / −	POSITIVE 4 YRS. AFTER	Only member with demonstratable antibodies in family of 4 hereditary spherocytoses.
7. V.D. ♀ Aged 30yrs.	+++ / −	++ / −	+ / −	− / −	− / −	− / −	− / −	NO	No active hemolysis: sisters to actively hemolysing patients; tested as routine.
8. S.R. ♀ Aged 35yrs.	+ / −	− / −	− / −	− / −	− / −	− / −	− / −	NO	

Family B

Fig. 1.

of whom manifested some degree of hypersplenic cytopenia. The 280 other individuals were either normal or had miscellaneous non-hematologic diseases. In the first group the developing serum test proved to be positive in 371 and the trypsinized red blood cell test was positive in 406 determinations. The sera of the second group were all negative and served as controls.

In the twenty-five cases of true hereditary spherocytosis studied in this series one or both tests were positive in eight cases (Fig. 1). The trypsinized red blood cell test was positive in six, the developing sera test was positive in four, and both were positive in two instances. No relationship could be established between the presence or titer of antibodies and the activity of the hemolytic process. There was also no consistent titer response to splenectomy. For example, both tests were equally positive in Case 1 before and after splenectomy, and in Cases 4, 5, and 6 at varying periods from four months to ten years after surgery and also in four patients without splenectomy. There were seven female patients and one male patient with demonstrable antibodies in this group.

In forty-eight of fifty-eight cases with acquired hemolytic anemia (Figs. 2 and 3) antibodies were found with one or both techniques. The trypsinized red blood cell test was positive in forty-three; the developing serum test was positive in forty-two. Both tests were positive in thirty-seven and both negative

in ten. The various etiologies of the acquired hemolytic anemias are listed in Table I. In this group a high degree of correlation between the two tests was evident. However, the relationship between the antibody titer and the intensity of the hemolytic process, while greater than was evident in the patients with hereditary spherocytosis, was not as predictable or as definite as others have reported for similar cases.[34, 35] The influence of splenectomy on the persistence of antibody was relatively the same for both groups.

TABLE I. ACQUIRED HEMOLYTIC ANEMIAS

ETIOLOGY	NUMBER OF CASES
Idiopathic	15
Leucemias	
Lymphatic	9
Monocytic	1
Myelogenous	1
Reticulum cell sarcoma	1
Letterer-Siwe's disease	1
Boeck's sarcoid	1
Hodgkin's syndrome	1
Infectious mononucleosis	1
Chronic inflammatory splenomegaly	1
Congestive splenomegaly	4
Chronic congestive splenomegaly (Banti's)	7
Chronic congestive splenomegaly (Felty's)	1
Cold agglutinins	2
Essential thrombocytopenic purpura (with mild hemolytic anemia)	1
Panhematopenia (secondary?)	1
Total	48

Six of nine cases of thalassemia (Fig. 4) showed demonstrable antibodies with one or both tests. Both tests were negative in three cases. Again there was no correlation between the severity of the anemia and the titer of antibodies. The tests were negative in two cases of sickle-cell anemia.

While the foregoing findings were at least suggestive of some relationship between the positive antibody tests and the hemolytic process, positive tests were also encountered in certain other blood dyscrasias without evidence of erythrocytic destruction. Included in the study of the hypersplenic syndromes were twenty-two cases of essential thrombocytopenic purpura (Fig. 5). In these patients, antibodies for trypsinized red cells were demonstrated in six, in five of whom no concomitant hemolytic process was discernible. The tests were negative in the remaining sixteen cases.

Because of the hyperglobulinemia in multiple myeloma, six cases were studied with these techniques. Both tests were positive in three instances, and in one the trypsinized red cell test alone was positive. No indications of hemolysis or other hypersplenic manifestations were present in any individuals of this group.

The positive benefit following removal of the spleen in most of the clinical hypersplenic entities focused our attention upon this organ as a potential source

ACQUIRED HEMOLYTIC ANEMIA

PERIPHERAL BLOOD TITERS WITH TRBC AND DEVELOPING SERUM (Coombs')

SUMMARY: TOTAL PATIENTS STUDIED: 58

POSITIVE TRBC : 43 | POSITIVE BOTH : 37

POSITIVE D.S. : 42 | NEGATIVE BOTH : 10

CASE	TITER : TRBC = + D.S. = +									COMMENT	
	U	2	4	8	16	32	64	128	256	RE: SPLENECTOMY	ETIOLOGY
9. J.K. ♂ Aged 33 yrs.	++++ / ++++	++++ / ++++	++++ / ++++	+++ / ++	+++ / ++	+++ / ++	++ / +	++	++	POSITIVE 5 YEARS AFTER	IDIOPATHIC
10. W.T. ♂ Aged 21 yrs.	++ / ++++	+ / ++++	− / ++++	− / +++	− / ++	− / ++	− / ++	− / ++	− / +	POSITIVE 1 YEAR AFTER	IDIOPATHIC
11. G.W. ♂ Aged 1½ yrs.	++++ / +	+++ / −	++ / −	+ / −	+ / −	− / −	− / −	−		POSITIVE 2 MONTHS AFTER	IDIOPATHIC
12. A.P. ♀ Aged 63 yrs.	++++ / ++++	+++ / ++	++ / +	++ / −	− / −	− / −	− / −	− / −	− / −	POSITIVE 2 YEARS AFTER	IDIOPATHIC
13. E.B. ♂ Aged 69 yrs.	++++ S / ++++ S	++++ S / ++++	++++ / ++++	++++ / ++++	+++ / ++++					POSITIVE BEFORE & AFTER	IDIOPATHIC
14. H.B. ♂ Aged 69 yrs.	++++ / ++	+++ / +	+ / +	− / +	− / +	− / −	− / −			NO	IDIOPATHIC
15. L.R. ♀ Aged 81 yrs.	+++ / +	++ / −	+ / −	− / −	− / −	− / −	− / −	− / −		POSITIVE 4 MONTHS AFTER	IDIOPATHIC
16. S.L. ♀ Aged 24 yrs.	++++ / ++++	++++ / ++++	++ / +++	+ / ++	− / +	− / −	− / −	− / −		POSITIVE 6 MONTHS AFTER	IDIOPATHIC
17. T.W. ♀ Aged 60 yrs.	++ / +	+ / +	− / −	− / −	− / −	− / −	− / −	− / −		POSITIVE 5 MONTHS AFTER	IDIOPATHIC
18. W.J. ♂	+++ / −	++ / −	+ / −	− / −	− / −	− / −	− / −	− / −		NO	IDIOPATHIC
19. F.F. ♂ Aged 49 yrs.	− / ++	− / +	− / +	− / −	− / −	− / −	− / −			NO	IDIOPATHIC
20. P.R. ♀ Aged 65 yrs.	+++ / +	+ / ++	+ / ++	± / ++	− / ++					NO	IDIOPATHIC
21. M.D. ♀ Aged 81 yrs.	+++ / +	++ / +	+ / +	− / +	− / −					NO	IDIOPATHIC
22. D.W. ♀ Aged 72 yrs.	++++ S / +++	++++ / ++	+++ / +	++ / +	+ / −					NO	IDIOPATHIC
23. M.S. ♀ Aged 21 yrs.	++++ S / ++++	++++ / +++	++++ / ++	+++ / ++	++ / +	+ / −	− / −	− / −	−	POSITIVE BEFORE & AFTER	IDIOPATHIC

Fig. 2.

of the observed immunologic phenomena. Comparative studies of circulating peripheral and splenic antibodies were made in thirty cases at the time of splenectomy (Fig. 6). In ten instances before the spleen was removed, splenic arterial and venous blood samples were obtained before *(tidal blood)* and after *(reserve blood)* splenic contraction. In all cases a manually expressed specimen of splenic blood *(residual blood)* was obtained immediately after surgical removal of the epinephrine-contracted spleen. In the twenty-four individuals showing demonstrable antibody in the *splenic residual blood*, only nineteen had antibodies also in the circulating peripheral blood. In the six cases in which antibody was not found in the splenic blood, none was detected in the peripheral blood. Of particular interest in this series were the five cases in which antibody was detected in the splenic specimens and none was found in the peripheral blood (Fig. 6, Cases 50, 68, 69, 70, and 71). The titers of the *splenic residual blood* were generally higher and quantitative agglutination in the various dilutions was more complete.

ACQUIRED HEMOLYTIC ANEMIA (Continued)

PERIPHERAL BLOOD TITERS WITH TRBC AND DEVELOPING SERUM (Coombs')

CASE	TITER									COMMENT	
	U	2	4	8	16	32	64	128	256	RE: SPLENECTOMY	ETIOLOGY
24. L.B. ♀ Aged 56 yrs.	++ / ++++	++ / +++	+ / ++	+ / ++	+ / +	− / −	− / −			POSITIVE 1½ YEARS AFTER	CHRONIC LYMPHATIC LEUKEMIA
25. V.H. ♀ Aged 61 yrs.	++++ / ++	+ ± / +	+ / −	− / −	− / −					POSITIVE BEFORE & AFTER	CHRONIC LYMPHATIC LEUKEMIA
26. A.S. ♀ Aged 61 yrs.	− / ++ ±	− / +	− / −	− / −	− / −					POSITIVE 2 MONTHS AFTER	CHRONIC LYMPHATIC LEUKEMIA
27. M.P. ♂ Aged 72 yrs.	++++ / ++++	++++ / +++	+++ / ++	++ / ++	+ / −	± / −	− / −			POSITIVE BEFORE & AFTER	CHRONIC LYMPHATIC LEUKEMIA
28. C.R. ♂ Aged 75 yrs.	++ ± / ++++	++ / ++++	++ / +++	+ / ++	+ / +	−	−	−	−	POSITIVE BEFORE & AFTER	CHRONIC LYMPHATIC LEUKEMIA
29. S.W. ♀ Aged 52 yrs.	++++ / +++	+++ / ++	++ / +	+ / −	− / −					POSITIVE BEFORE & AFTER	CHRONIC LYMPHATIC LEUKEMIA
30. D.H. ♀ Aged 23 yrs.	++++ S / −	++++ / −	+++ / −	+++ / −	++ / −	± / −	− / −	− / −	− / −	NO	LYMPHATIC LEUKEMIA
31. E.G. ♀ Aged 69 yrs.	+++ / +++	++ / ++	++ / ++	+ / +	− / +	−	−			POSITIVE BEFORE & AFTER	CHRONIC LYMPHATIC LEUKEMIA
32. H.R. ♂ Aged 51 yrs.	++++ / −	+++ / −	+++ / ±	++ / ++	+ / ++					NO	CHRONIC LYMPHATIC LEUKEMIA
33. E.B. ♀ Aged 54 yrs.	− / ++	− / −	− / ±	− / −	− / −	− / −	− / −	− / −		NO	MONOCYTIC LEUKEMIA
34. O.R. ♂ Aged 56 yrs.	+++ / +++	++ / ++	+ / +	+ / ±	− / −	− / −	− / −			POSITIVE 1½ YEARS AFTER	MYELOGENOUS LEUKEMIA
35. G.G. ♀ Aged 66 yrs.	++++ / + ±	+++ / +	+ ± / −	+ / −	± / −					NO	RETICULUM CELL SARCOMA
36. T.C. ♂ Aged 17 mos.	/ +++	TRBC	not done							POSITIVE BEFORE	LETTERER-SIWE'S DISEASE
37. H.S. ♀ Aged 60 yrs.	++ / ++ ±	++ / +	+ / +	+ / −	− / −					POSITIVE BEFORE	HODGKIN'S SYNDROME
38. S.T. ♀ Aged 20 yrs.	++ / ++	− / +	− / +	− / +	− / ±	− / −	− / −			POSITIVE BEFORE & AFTER	CHRONIC CONGESTIVE SPLENOMEGALY (Banti's)
39. T.P. ♀ Aged 45 yrs.	+++ / + ±	+ / +	− / −	− / −	− / −					POSITIVE BEFORE & AFTER	CHRONIC CONGESTIVE SPLENOMEGALY (Banti's)
40. I.T. ♀ Aged 26 yrs.	++ ± / + ±	++ / +	+ / −	± / −	− / −					POSITIVE BEFORE	CHRONIC CONGESTIVE SPLENOMEGALY (Banti's)
41. M.H. ♀ Aged 19 yrs.	+++ / +	++ / −	+ / −	± / −	− / −					POSITIVE BEFORE & NEGATIVE AFTER	CHRONIC CONGESTIVE SPLENOMEGALY (Banti's)
42. J.M. ♂ Aged 32 yrs.	+ / +	− / −	− / −	− / −	− / −					POSITIVE BEFORE & NEGATIVE AFTER	CHRONIC CONGESTIVE SPLENOMEGALY (Banti's)
43. G.M. ♀ Aged 24 yrs.	++ / −	+ / −	− / −	− / −	− / −	− / −				POSITIVE BEFORE & NEGATIVE AFTER	CHRONIC CONGESTIVE SPLENOMEGALY (Bantis)
44. R.A. ♂ Aged 16 yrs.	+ ± / −	± / −	− / −	− / −	− / −	−				POSITIVE BEFORE & NEGATIVE AFTER	CHRONIC CONGESTIVE SPLENOMEGALY (Banti's)
45. F.S. ♀ Aged 52 yrs.	++ ± / +	− / −	− / −	− / −	− / −					POSITIVE 1 YEAR AFTER	CHRONIC CONGESTIVE SPLENOMEGALY (Felty's)
46. R.B. ♀ Aged 41 yrs.	+++ / ++	++ / ±	+ / −	− / −	− / −	− / −				POSITIVE BEFORE & AFTER	CONGESTIVE SPLENOMEGALY (CARCINOMA)
47. W.T. Aged 19 yrs.	− / + ±	− / + ±	− / +	− / −	− / −	−				POSITIVE IMMEDIATELY AFTER	CONGESTIVE SPLENOMEGALY
48. E.L. ♂ Aged 68 yrs.	++ / −	+ / −	+ / −	+ / −	+ / −	− / −				POSITIVE BEFORE	CONGESTIVE SPLENOMEGALY
49. T.S. ♀ Aged 31 yrs.	++ / +	+ / ±	− / ±	− / −	− / −	− / −				POSITIVE 1 WEEK AFTER	BOECK'S SARCOID
50. G.C. ♀ Aged 49 yrs.	++ / +	− / −	− / −	− / −	− / −					NEGATIVE BEFORE, POSITIVE IMMEDIATELY AFTER	RHEUMATIC HEART DISEASE WITH FAILURE
51. C.G. ♂ Aged 55 yrs.	++++ S / ++++	++++ S / +++	++++ / ±	+++ / −	++ / −	±	−	−	−	NO	COLD AGGLUTININS
52. L.M. ♂ Aged 67 yrs.	++++ S / ++++ S	++++ S / ++++ S	++++ S / ++++	++++ / ++++	++++ / ++	++ / ++	++ / +	+ / +	− / ±	POSITIVE BEFORE & AFTER	COLD AGGLUTININS
53. D.C. ♂ Aged 71 yrs.	+++ / −	++ / −	− / −	− / −	− / −					NO	CHRONIC INFLAMMATORY SPLENOMEGALY
54. M.C. ♂ Aged 22 yrs.	++ / ±	+ / −	± / −	− / −	− / −					NO	INFECTIOUS MONONUCLEOSIS
55. D.L. ♀ Aged 24 yrs.	+++ ± / +	++ / +	− / +	− / ±	− / −					POSITIVE BEFORE & AFTER	ESSEN. THROMB. PURPURA (with mild hemolytic anemia)
56. R.C. ♀ Aged 5 yrs.	+ ± / +	+ / ±	− / −	− / −	− / −					NO	PANHEMATOPENIA (Secondary ?)

Fig. 3.

The foregoing positive findings when contrasted with the completely negative results in 388 persons with no evidence of hemolytic anemia add significance to the suspected relationship which these antibodies may have to erythrocytic destruction.

THALASSEMIA

PERIPHERAL BLOOD TITERS WITH TRBC AND DEVELOPING SERUM (Coombs')

SUMMARY: TOTAL PATIENTS STUDIED = 9

POSITIVE TRBC : 6	POSITIVE BOTH : 3
POSITIVE D.S. : 3	NEGATIVE BOTH : 3

CASE	TITER: TRBC = ✦ D.S. = + U	2	4	8	16	32	COMMENT RE: SPLENECTOMY	GENERAL	
57. Y.S. ♂ Aged 30 yrs.	✦ ± / +	± / +	− / −	− / −	− / −		NO	Patient: exacerbations ☇ moderate hemolysis since birth.	
58. M.P. ♀ Aged 51 yrs.	✦✦✦ / +	✦✦ / +	✦ / ±	− / +	− / −		NO	Mother: history pigment cholelithotomy; mild hemolysis	FAMILY 5
59. S.S. ♀ Aged 36 yrs.	✦✦ ± / −	✦ / −	± / −	− / −	− / −		NO	Sister: history cholecystectomy; mild hemolysis.	
60. V.P. ♀ Aged 31 yrs.	✦✦ / −	✦ / −	± / −	− / −	− / −		NO	Sister: history cholecystectomy; moderate hemolysis.	
61. S.A. ♀ Aged 21 mos.	✦✦ / ++	✦✦ / +	✦ / ±	± / +	− / −		POSITIVE BEFORE AND AFTER	Patient: splenectomy for hypersplenic thrombocytopenia; severe hemolysis.	FAMILY 4
62. P.A. ♂ Aged 32 yrs.	✦✦✦✦ / −	✦✦✦✦ / −	✦✦✦✦ / −	✦✦✦✦ / −	✦✦ / −	✦ / −	NO	Father; moderate hemolysis.	

Fig. 4.

ESSENTIAL THROMBOCYTOPENIC PURPURA

PERIPHERAL BLOOD TITERS WITH TRBC AND DEVELOPING SERUM (Coomb's)

SUMMARY: TOTAL PATIENTS STUDIED = 22

POSITIVE TRBC : 6	POSITIVE BOTH : 6
POSITIVE D.S. : 6	NEGATIVE BOTH : 16

CASE	TITER: TRBC = ✦ D.S. = + U	2	4	8	16	COMMENT RE: SPLENECTOMY	GENERAL
63. J.H. ♀ Aged 34 yrs.	✦✦✦ / +	✦✦ / +	✦ / +	− / −	− / −	POSITIVE BEFORE AND AFTER	
64. Y.B. ♂ Aged 17 yrs.	✦✦ / ++	− / −	− / −	− / −	− / −	POSITIVE BEFORE AND AFTER	
65. E.E. ♀ Aged 34 yrs.	✦ ± / +	− / −	− / −	− / −	− / −	POSITIVE BEFORE AND AFTER	
66. A.M. ♀ Aged 35 yrs.	✦✦✦✦ / ++++	✦✦✦ / ++ ±	✦✦ / ++	✦✦ / +	✦ / +	POSITIVE BEFORE AND AFTER	A generalized reticulo-endothelial hyperphagocytosis; splenectomy failed to establish re-equilibration.
67. M.C. ♀ Aged 33 yrs.	✦✦ / ++	✦ / +	✦ / +	− / −	− / −	POSITIVE AFTER	A generalised reticulo-endothelial hyperphagocytosis; splenectomy failed to establish re-equilibration.
55* D.L. ♀ Aged 24 yrs.	✦✦✦ ± / +	✦✦ / ++ ±	− / ++	− / ++ ±	− / +	POSITIVE BEFORE AND AFTER	Accompanying mild Hemolytic Anemia. Also included in Figure 3

* Double inclusion Fig. 3

Fig. 5.

COMPARATIVE STUDIES OF PERIPHERAL AND SPLENIC BLOOD TITERS WITH TRBC AND DEVELOPING SERUM IN HEREDITARY SPHEROCYTOSIS AND ACQUIRED HEMOLYTIC ANEMIA

SUMMARY: TOTAL PATIENTS STUDIED: 30

SPLENIC BLOOD	PERIPHERAL BLOOD
Positive TRBC : 23 with	Positive TRBC : 17
	Negative TRBC : 7
Positive D.S. : 18 with	Positive D.S. : 15
	Negative D.S. : 9
Positive Both : 17 with	Positive Both : 3
	Negative Both : 5
Negative Both : 6 with	Positive Both : 0
	Negative Both : 6

$VEIN_1$ = before epinephrine
$VEIN_2$ = after

Case	BLOOD OBTAINED FROM:	TITER : TRBC = + D.S. = + U	2	4	8	16
68	PERIPHERAL BLOOD					
	RESIDUAL SPLENIC BLOOD					
	SPLENIC $VEIN_1$					
	SPLENIC $VEIN_2$					
69	PERIPHERAL BLOOD					
	RESIDUAL SPLENIC BLOOD					
	SPLENIC ARTERY					
	SPLENIC $VEIN_1$					
	SPLENIC $VEIN_2$					
70	PERIPHERAL BLOOD					
	RESIDUAL SPLENIC BLOOD					
	SPLENIC ARTERY					
	SPLENIC $VEIN_1$					
	SPLENIC $VEIN_2$					
71	PERIPHERAL BLOOD					
	RESIDUAL SPLENIC BLOOD					
	SPLENIC ARTERY					
	SPLENIC $VEIN_1$					
	SPLENIC $VEIN_2$					
1	PERIPHERAL BLOOD					
	SPLENIC ARTERY					
	SPLENIC $VEIN_1$					
	SPLENIC $VEIN_2$					
23	PERIPHERAL BLOOD	S				
	RESIDUAL SPLENIC BLOOD					
	SPLENIC ARTERY					
	SPLENIC $VEIN_1$					
	SPLENIC $VEIN_2$					
25	PERIPHERAL BLOOD					
	RESIDUAL SPLENIC BLOOD	S				
	SPLENIC ARTERY					
	SPLENIC $VEIN_1$					
	SPLENIC $VEIN_2$					
27	PERIPHERAL BLOOD		S			
	RESIDUAL SPLENIC BLOOD	S				
28	PERIPHERAL BLOOD					
	RESIDUAL SPLENIC BLOOD					

Case	BLOOD OBTAINED FROM:	TITERS U	2	4	8	16
29	PERIPHERAL BLOOD					
	RESIDUAL SPLENIC BLOOD					
	SPLENIC ARTERY					
	SPLENIC $VEIN_1$					
	SPLENIC $VEIN_2$					
31	PERIPHERAL BLOOD					
	RESIDUAL SPLENIC BLOOD					
37	PERIPHERAL BLOOD					
	RESIDUAL SPLENIC BLOOD					
38	PERIPHERAL BLOOD					
	SPLENIC ARTERY					
	SPLENIC $VEIN_1$					
	SPLENIC $VEIN_2$					
39	PERIPHERAL BLOOD					
	RESIDUAL SPLENIC BLOOD	S				
40	PERIPHERAL BLOOD					
	RESIDUAL SPLENIC BLOOD					
42	PERIPHERAL BLOOD					
	RESIDUAL SPLENIC BLOOD					
43	PERIPHERAL BLOOD					
	RESIDUAL SPLENIC BLOOD					
44	PERIPHERAL BLOOD					
	RESIDUAL SPLENIC BLOOD					
46	PERIPHERAL BLOOD					
	RESIDUAL SPLENIC BLOOD					
47	PERIPHERAL BLOOD					
	RESIDUAL SPLENIC BLOOD					
48	PERIPHERAL BLOOD					
	RESIDUAL SPLENIC BLOOD					
50	PERIPHERAL BLOOD					
	RESIDUAL SPLENIC BLOOD					
52	PERIPHERAL BLOOD	S	S	S	S	S → 2048
	RESIDUAL SPLENIC BLOOD	S	S	S	S	S → 8912
	SPLENIC ARTERY	S	S	S	S	S → 8192
	SPLENIC $VEIN_1$	S	S	S	S	S → 1024
	SPLENIC $VEIN_2$	S	S	S	S	S → 4000
55	PERIPHERAL BLOOD					
	RESIDUAL SPLENIC BLOOD					

Fig. 6.

It would seem from these considerations that antibodies for red cells detectable by the two methods employed are present in 47 per cent of the cases of hypersplenism as defined. Thus, it is possible to demonstrate accompanying antibodies in a considerable number of these conditions, which, although playing as yet an unknown clinical role, are in all other respects similar to the incomplete type anti-Rh antibody. This latter antigen has been ruled out as affecting the agglutinations in all but three instances in the present study. In three additional cases the antibody could be traced directly to sensitization by incompatible transfused blood cells.

DISCUSSION

The differentiation between acquired hemolytic anemia and hereditary spherocytic hemolytic anemia is important from the prognostic standpoint, but is frequently difficult to establish with routine clinical and laboratory methods. Elimination of all splenic tissue in hereditary spherocytosis will permanently correct the hemolytic anemia in over 98 per cent of all cases. On the other hand, splenectomy in acquired hemolytic anemia is much less effective in abolishing the clinical manifestations. The clinical picture, the quantitative studies of the peripheral blood and bone marrow elements, and the osmotic and mechanical erythrocyte fragilities may be very similar in both syndromes. Shen, Castle, and Fleming[36] have pointed out a characteristic of erythrocytes in hereditary spherocytosis, viz. a marked accentuation of the mechanical fragility after incubation, as a means of differentiation. While the possibility of differential diagnosis by demonstration of autoagglutinins in the acquired hemolytic anemias has been emphasized in recent years, the concept, as Neber and Dameshek have cited,[15] was initially advocated by Widal, Abrami, and Brulé as early as 1908. The current claims, however, for developing (Coombs) serum[11, 12] as a reliable differential diagnostic procedure have not been verifiable in our laboratory, since 32 per cent of our patients (eight cases) with proved hereditary spherocytosis have shown incomplete antibodies by one or both methods. The three cases of hereditary spherocytosis with demonstrable incomplete hemagglutinins, as reported from other laboratories, were in acute hemolytic crisis.[14, 15, 19, 21] In our series there was no direct relationship demonstrated between the presence or quantitative titers of antibodies and the hemolytic process.

The sera from seventy (38 per cent) of the 185 patients studied with various hematologic dyscrasias showed a positive trypsinized red blood cell test, while sixty (32 per cent) had antibody globulins detected on the red blood cell by the developing serum test; in seventy-seven or 42 per cent, one or both tests were positive. Of the seventy-seven patients in whom one or both tests were positive, sixty-eight cases, or *88 per cent, manifested either active hemolysis or had had a recent history of associated hemolytic phenomenon, or exhibited an inherited trait of defective erythrocytes.* Of the remaining nine instances with one or both tests positive, five were essential thrombocyopenic purpura (Fig. 5) and four were multiple myeloma. Only one of a total of six cases of essential

thrombocytopenic purpura showing positive tests had concomitantly associated hemolysis (Case 55, Figs. 3 and 5); no evidence of hemolysis was demonstrable in the remaining five instances.

The evidence so far indicates that the antibodies demonstrated with these techniques have probably been primarily associated with the hemolytic phenomenon. The positive findings, therefore, obtained in clinically nonhemolytic patients are of particular interest. Increasing evidence in recent years has led us to regard essential thrombocytopenic purpura (Werlhof's disease) as a specific hypersplenic manifestation. In hypersplenic syndromes the type and quantity of circulating elements involved have been observed to change from time to time.[39, 41] For example, in the same patient the hypersplenic mechanism may be manifested by an acute hemolytic crisis at one time, and on another occasion a thrombocytopenic purpura may develop without any hemolytic component. Varying degrees of anemia, thrombocytopenia, and granulocytopenia, pure or in combination, have been encountered repeatedly clinically. The presumptive evidence that these patients have at some time experienced erythrocyte sensitization is in line with recent concepts. It is a rare situation when removal of all splenic tissue does not correct the thrombocytopenia in a patient with essential thrombocytopenic purpura. "Generalized reticuloendothelial hyperphagocytosis" occurred in two (Fig. 5; Cases 66 and 67) of our twenty-two cases of essential thrombocytopenic purpura studied in this series, and in both of these patients incomplete antibodies for erythrocytes were demonstrated with both techniques, but no hemolytic component was evident. Recently a thrombotic thrombocytopenic purpura with a positive developing serum (Coombs) test was reported by Singer and associates.[27] Whether autosensitization to specific antigens from platelets and granulocytes occurs remains a problem for the future development of adequate methods for their recognition and detection.

What relationship can these incomplete antibodies for erythrocytes bear to the pathologic cytopenic states associated with the spleen? From these data it becomes apparent that circulating antibodies can be demonstrated with the newer techniques not only in 83 per cent of the acquired hemolytic anemias and 32 per cent of the hereditary spherocytoses, but also in 27 per cent of the thrombocytopenic purpuras and in 66 per cent of the thalassemias. Hereditary spherocytosis and essential thrombocytopenic purpura are regarded as *primary* hypersplenic syndromes. A varying degree of splenic involvement, i.e. *secondary* hypersplenism, may be manifested in acquired hemolytic anemia and in thalassemia, though rarely. In the study of the hypersplenic cases which allowed comparison of peripheral antibody titers with the splenic blood titers at the time of splenectomy, there were eighteen patients in whom antibody concentrations were found to be higher in the *splenic* blood (Fig. 6). Even stronger positive evidence pointing to the hyperactive spleen as the source of these hemagglutinins is found in the five cases in which incomplete antibodies were demonstrable only in the *splenic residual blood*, and not in the peripheral blood.

On the other hand are the six cases in which no antibody was present in either the peripheral blood or the splenic blood. The four cases of multiple

myeloma without evidence of hypersplenism also await adequate explanation. One can postulate a possible delayed antibody production in a short but intense clinical episode or a genetically modified immunologic capacity as explanations in the former group. The pathologic globulin elevations of multiple myeloma are in all probability associated in some way with the positive findings there. These questions along with the lack of a consistent correlation between the presence or titer of antibody and the clinical evidence of active hemolysis, and the persistence of appreciable agglutinin titers after splenectomy, but with prompt clinical cessation of all signs and symptoms, will undoubtedly be better understood when the precise role of antibodies in the various diseases studied herein is clarified.

As usually understood, antibody in vivo is an antigenically modified globulin, produced by the reticuloendothelial system of phagocytic cells, and/or lymphocytes, and/or plasmacytes, which globulin has a specific protective immune function. In vitro measurements of agglutinins, precipitins, opsonins, etc., do not necessarily indicate the measure of the protective function. Antibody titers determined in vitro may fluctuate over a wide range without any consistent parallel in vivo effect.

Autosensitization is not a new concept considering Ehrlich's early postulation of the state of "horror autotoxicus"; and specific sensitization by ordinarily nonantigenic substances through combination with the altered protein of host cells has been frequently suggested. Aside from the cold-agglutinin, it has been only recently that evidence has been presented indicating autoimmunization with erythrocytes.

The newer data revealed with the recently devised techniques are requiring a revision of our concepts and thus of our investigative approach to many hematologic problems. Incomplete antibodies will not agglutinate untreated, normal cells in vitro. However, they are specific not only for the erythrocytes of the individual producing them but for normal erythrocytes in general, this specific combination in vitro or in vivo being demonstrable by developing serum (Coombs). Erythrocytes "optimally" treated with trypsin are agglutinated directly in saline by serum containing these antibodies but not by normal serum. If the enzymatic procedure is carried beyond this "optimum," agglutination is produced by most normal sera, indicating possibly the widespread presence of normally occurring antibodies for enzymaticaly modified erythrocytes. Such antibodies for red blood cells in normal sera modified by various viral, bacterial, and plant enzymes have been described by Thomsen,[42] Burnet and Anderson,[43] Pickles,[44] Wheeler,[6, 7] and Moskowitz and Treffers.[45] Interesting from the present viewpoint is the fact that these antibodies are apparently not specific for normal, untreated cells, as are those from autoimmunized persons, since in all studies using the developing serum technique, normal cells tested directly after washing, or after having been treated with normal serum, do not agglutinate and in fact are used as controls.

The facts based on the unique opportunity presented by the spleen's characteristic anatomic, physiologic, and immunologic potentialities would seem to

warrant the hypothesis that the spleen can and very probably does play a special role in the production of autohemagglutinins. An accentuation of its normal reservoir function for blood cells may result from any of a number of causes that derange the delicate vascular sinusoidal-parenchymal equilibrium, resulting in an alteration in the number and type of those elements withheld from the general circulation. Another normal splenic function is that of removing from the peripheral circulation all defective, damaged, or senile cells; any increase in debilitated cells requiring removal would engorge the splenic sinuses and stimulate phagocytosis by the reticuloendothelial macrophages.

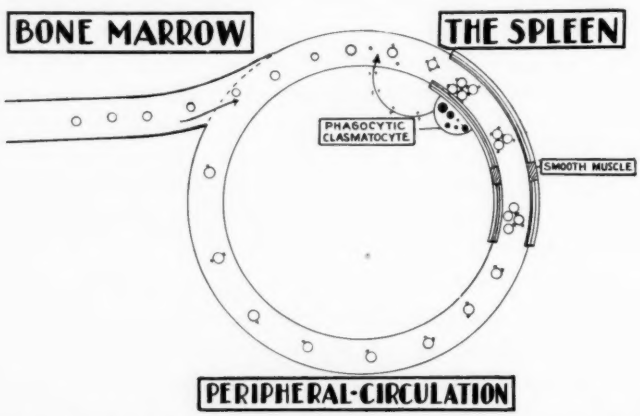

Fig. 7.—*A hypothesis of the immunologic phases of hypersplenism*: A slowing of the circulation through the spleen by any of a number of causes may upset the delicate vascular equilibrium and *reserve blood* balance and cause changes in the quality and integrity and therefore, the stability of the withheld formed elements to the degree that facilitates their phagocytosis by the reticuloendothelial macrophages. These cells have been shown to be at least one source of and to play a major role in antibody synthesis. With the increased antigenic material being supplied, the production of antibodies mounts. They enter the circulation, sensitize other freshly delivered normal cells from the bone marrow, and these newly autosensitized cells agglutinate and then become more easily withheld in the sluggish circulation of the splenic vascular beds, establishing a vicious destructive cycle.

Sabin[46] and Houghton[47, 48] have been among those who have confirmed the fact that these clasmatocytes (macrophages) are one source of antibody in the animal organism, or at least play some major role in antibody formation. With the increased antigenic stimulation provided by these hypersequestered cells, the result may well be an increased production of antibodies, which, upon entering the general circulation, sensitize still more normal erythrocytes (Fig. 7). These cells, sensitized by incomplete antibody, and exposed in turn to the hemoconcentrating and deplasmerizing effect of the splenic sinusoids, find this environment conducive to weak agglutination. Thus a vicious cycle is initiated originally, either by local circulatory stasis or by a defective cell type withheld and incorporated within the *splenic reserve* and/or *residual blood*. Any increased activity of the immunologic mechanisms may accentuate and perpetuate the cycle. The process having been started in the recesses of the spleen, it may in some instances result in still further elaboration of specific cellular agglutinins in other organs and tissues in which reticuloendothelial cells are capable of this immunologic reaction—for example the Kupffer cells of the liver sinuses.

Threshold antigenic doses of the modified cells may escape the splenic congestion and in the peripheral circulation provide the opportunity for other reticuloendothelial phagocytic elements to remove the increasing number of sensitized erythrocytes. Eliminating the other mechanisms involved in some of the hemolytic phenomena, these patients with "pure" hypersplenic cytopenia, as here defined, may anticipate complete hematologic and clinical re-equilibrium after removal of all splenic tissue. The benefit of splenectomy may be due in part to the removal of a large sinusoidal vascular bed favorable to "cell sludge," and in part to the removal of the largest localization of phagocytic, antibody-forming cells. This cytoimmunologic concept explains the therapeutic failure and recurrences of excessive cell destruction despite compensatory marrow hyperplasia following splenectomy, in terms of (1) accessory splenic tissue still present and (2) hyperimmune circulating antibodies stimulating generalized extrasplenic reticuloendothelial hyperplasia and hyperphagocytosis again in a vicious cycle, at present unaffected favorably by any known medical or surgical measures.

SUMMARY

A study of incomplete antibodies with the developing serum (Coombs) and the trypsinized red blood cell techniques has been made in a series of hemolytic anemias and other hematologic dyserasias.

This study has failed to confirm the validity of the developing serum (Coombs) test as a reliable diagnostic procedure to differentiate acquired hemolytic anemia and hereditary spherocytosis.

Of seventy-seven patients with positive tests, sixty-eight (88 per cent) manifested either an active hemolysis, had a recent history associated with the hemolytic phenomenon, or exhibited an inherited trait of defective erythrocytes.

Of the nine patients (12 per cent) with one or both tests positive and exhibiting no hemolysis, five had essential thrombocytopenic purpura. A previous erythrocyte sensitization is hypothesized and is in line with recent concepts of the hypersplenic phenomena. The four remaining positive nonhemolytic cases were multiple myeloma; this may be related to the hyperglobulinemia.

Comparative studies of circulating peripheral and splenic antibodies were made in thirty cases at the time of splenectomy. The generally higher titers and quantitative agglutination in the various dilutions of the *splenic residual blood* focus consideration on the spleen as the initiating source and perpetuator of the autosensitizing antibodies.

The relationship of these incomplete antibodies to erythrocytes, granulocytes, and thrombocytes in the various pathologic cytopenic states associated with hypersplenic activity is hypothesized and discussed.

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THE ANEMIA OF CANCER PATIENTS AND ITS RELATION TO METASTASES TO THE BONE MARROW

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INTRODUCTION

THE terms myelopathic anemia, osteosclerotic anemia, chronic nonleucemic myelosis, leucoerythroblastic anemia, and myelophthisic anemia are applied to the anemia allegedly resulting from morphologic replacement of the bone marrow by neoplastic or connective tissue.[1, 2, 3, 4] It is usually considered that anemia in cancer patients, excluding that obviously due to blood loss or, in rare instances, to hemolysis, is due to bone marrow invasion. This mechanical explanation does not, however, gain the credence of certain competent authorities,[5, 6] who point out that the unaffected areas of the marrow do not take over the compensatory activity which might be expected on the basis of this theory. Furthermore, we have frequently encountered cancer patients with anemia in whom no underlying pathologic condition of the skeleton was present, and, conversely, we have encountered patients with extensive metastases to bone marrow who demonstrated no anemia.

These simple contradictory clinical observations and the lack of agreement in the literature concerning the etiology of anemia associated with cancer led us to question whether or not the mechanical explanation of anemia in these patients was wholly correct. Accordingly, the following studies were made. (1) The relation of the incidence and severity of anemia to bone marrow metastases was studied by analysis of the morphologic data concerning the peripheral blood and by correlation of these data with bone marrow involvement in 193 patients with advanced carcinoma. (2) A hematopoietic agent, cobaltous chloride, was given orally to selected patients, with and without metastases to bone, in order to ascertain whether the bone marrow of these patients was capable of increased red cell production.

The purpose of this paper is to report the results of these studies and to attempt to evaluate the relation of anemia to metastatic invasion of the bone marrow.

For simplicity, the anemias observed in this study were classified as follows.

(1) *Anemia due to blood loss.* These patients had either occult blood in the stool, demonstrable by the guaiac test, or a recent history of definite blood loss.

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(2) *Anemia due to nutritional deficiency.* These patients either responded to specific nutritional therapy or demonstrated a spontaneous return to normal hemoglobin levels after a period of hospitalization.

(3) *Anemia due to hemolysis.* These patients had either a positive Coombs test or an increase in the osmotic or mechanical fragility of their red cells together with reticulocytosis, hyperbilirubinemia, and an increased output of blood pigments.

(4) *Anemia due to myelopathy.* These patients had either metastases to bone, demonstrable by marrow aspiration or by x-ray, or else had no characteristics which would permit classification in any of the preceding categories.

(5) *Anemia due to myelopathy and to blood loss.* These patients had an anemia out of proportion to the amount of blood lost, or the anemia persisted long after bleeding ceased, or there was evidence of osseous metastases in addition to blood loss.

For the practical purposes of statistical analysis, the degree of anemia was also arbitrarily divided into three groups. A hemoglobin above 80 per cent was considered normal. Those anemias with a hemoglobin of 70 to 80 per cent were classified as mild, with a hemoglobin of 55 to 69 per cent as moderate, with a hemoglobin of less than 55 per cent as severe.

METHODS

The red and white cell counts were made on oxalated venous blood with U. S. Bureau of Standards pipettes and counting chambers. The hemoglobin was determined with an Evelyn photoelectric colorimeter, using 15.6 grams of hemoglobin per 100 c.c. as 100 per cent. Hematocrit determinations and red cell indices were performed by the method of Wintrobe, and the icterus indices were determined by comparing plasma with dichromate standards. Reticulocytes were counted on cover slips previously coated with a film of brilliant cresyl blue (0.5 per cent) dissolved in 95 per cent alcohol. After the film was dry, a fresh drop of blood was spread upon the cover slip in the usual manner and was thereafter fixed and counterstained with Wright's stain. The percentage of reticulocytes was then estimated by a count of at least 1,000 red blood cells under the oil immersion lens. The absolute number of reticulocytes was calculated by multiplying the total red blood count by the per cent of reticulocytes.

In order to detect the presence of hemolytic processes, the osmotic fragility of the erythrocytes was determined quantitatively by the method of Shen, Ham, and Fleming[7] and the mechanical fragility by the method of Shen, Fleming, and Castle.[8] Coombs tests were also made by the method of Coombs, Mourant, and Race.[9] Two drops of a 5 per cent suspension of the patient's washed red blood cells in normal saline were added to an equal volume of rabbit antihuman globulin serum. The sample was then incubated for one hour at 37° C. A drop from each sample was carefully pipetted from the bottom of the tube and was examined for agglutination under the microscope. If it were found to be positive, the titers of the direct Coombs test and the indirect Coombs test[10] were done as follows.

Two drops of the 5 per cent suspension of red blood cells were incubated at 37° C. for one hour with 2 drops from each tube of a series of saline dilutions of the antihuman serum rabbit serum. Thereafter, each red blood cell suspension was examined microscopically for evidence of agglutination. The red blood cells used for the indirect Coombs tests were obtained from normal individuals of group O. These cells, after being washed three times in physiologic saline and made up to a 5 per cent suspension in physiologic saline, were sensitized with aliquots of the patient's serum which had been adjusted to pH's of approximately 6.5, 7.0, and 7.8 according to the method of Gardner.[11] After sensitization the procedure used for the direct Coombs test was followed.

Occult blood in the stool was sought by the guaiac test.

Bone marrow puncture was made in most instances in the iliac crest near the anterior superior spine and in some instances in the midsternum at the level of the second interspace.

On each of the 193 patients a complete blood count was done on admission and on several occasions thereafter. On those patients with anemia, the additional hematologic studies already described, including bone marrow aspirations, were done. During the control periods and during periods of therapy, frequent red and white cell counts, hemoglobin determinations, hematocrits, reticulocyte counts, and icterus indices were performed. Bone marrow punctures were done at appropriate times in order to determine the comparative effects of the cobalt therapy on the marrow cytology.

During the control period of not less than five days, and in many instances over forty days, and during the periods of hematopoietic therapy, reticulocyte counts were done daily and complete blood counts at least every other day.

RESULTS OF STATISTICAL ANALYSIS

Relation of Anemia to Osseous Metastases.—Table I shows that of the 193 cancer patients seventy-seven (39.9 per cent) had a normal level of hemoglobin and 116 (60.1 per cent) had a definite anemia. Of the 116 patients with anemia, the anemia of fifty-two (44.9 per cent) was of mild degree, of forty-two (36.2 per cent) of moderate degree, and of twenty-two (19.0 per cent) of severe degree. Furthermore, of the seventy-seven patients without anemia, twenty-six (33.8 per cent) had evidence of bony metastases; whereas of the 116 patients with anemia, in only twenty-four (20.7 per cent) could such evidence be found. Conversely, in the fifty patients in whom metastsases were evident, only twenty-four (48 per cent) had anemia, as contrasted with the incidence of anemia in ninety-two (64.3 per cent) of the 143 patients without metastases. These data would seem to imply that the incidence of metastases to bone marrow is certainly no greater in cancer patients with anemia than in cancer patients without anemia. Even more pertinent, it would appear that the incidence of anemia is no greater in the presence of metastases than in their absence.

TABLE I. INCIDENCE OF ANEMIA IN 193 PATIENTS WITH ADVANCED CANCER AND THE RELATION OF ANEMIA TO BONY METASTASES

	MILD HGB. 70-80%		MODERATE HGB. 55-69%		SEVERE HGB. BELOW 55%		TOTAL CASES	
	NO.	%	NO.	%	NO.	%	NO.	%
No anemia: 77 cases (39.9%)								
Metastases to bone present							26	33.8
Metastases to bone absent							51	66.2
Total							77	100.0
Anemia present: 116 cases (60.1%)								
Metastases to bone present	14	12.1	6	5.2	4	3.5	24	20.7
Metastases to bone absent	38	22.8	36	31.0	18	15.5	92	79.3
Total	52	34.9	42	36.2	22	19.0	116	100.0

Classification of Anemia Associated With Cancer.—In Table II the 116 cases of anemia are classified according to the criteria described. Sixty-five (56.0 per cent) of the 116 cases are classified as myelopathic, fifteen (12.9 per cent) as myelopathic complicated by blood loss, and thirty-three (28.5 per cent) as due to blood loss alone. Only three (2.6 per cent) were due to hemolysis. No case of nutritional anemia was encountered. It should be noted that of the twenty-two instances of severe anemia, eighteen were associated with blood loss; and of the sixty-five cases classified as myelopathic uncomplicated by blood loss, only three were severe. It would appear, then, that severe anemia in a cancer patient is usually due to blood loss, and that anemia of the myelopathic type is not usually severe. Moreover, of the sixty-five cases of anemia of the myelopathic type not associated with blood loss, forty-three yielded no evidence of osseous metastases. This suggests that even when the cases due to blood loss or hemolysis are excluded, anemia does not occur more frequently in cancer patients with metastases to bone than it does in cancer patients without such metastases.

TABLE II. CLASSIFICATION OF 116 CASES OF ANEMIA FOUND IN CANCER PATIENTS

	MILD HGB. 70-80%		MODERATE HGB. 55-69%		SEVERE HGB. BELOW 55%		TOTAL CASES	
TYPE OF ANEMIA	NO.	%	NO.	%	NO.	%	NO.	%
Blood loss	13	11.2	11	9.5	9	7.8	33	28.5
Hemolytic	2	1.7	0	0	1	0.86	3	2.6
Myelopathic	36	31.0	26	22.4	3	2.6	65	56.0
Bony metastases present	14	12.1	6	5.2	2	1.7	22	18.9
Bony metastases absent	22	18.9	20	17.2	1	0.86	43	37.1
Myelopathic and blood loss	1	0.86	5	4.3	9	7.8	15	12.9
Bony metastases present	0	0	0	0	2	1.7	2	1.7
Bony metastases absent	1	0.86	5	4.3	7	6.1	13	11.2
Total	52	44.8	42	36.2	22	19.1	116	100

Morphology of Anemia Associated With Cancer.—Table III shows the distribution of mean corpuscular values among these 193 cancer patients. It may be seen that in the myelopathic group of anemias, hypochromia and microcytosis are definitely less pronounced than in the anemias associated with blood loss. The reticulocyte counts were normal or very slightly elevated. Very rarely, nucleated red blood cells appeared in the peripheral blood; if they were present, the anemia was usually severe. White cell counts were occasionally slightly increased. Such increases usually were accompanied by a polymorphonuclear leucocytosis and a shift to the left, or clinically by bouts of fever, and were presumed to be due to infection or necrosis of neoplastic tissue. The plasma pigments were not increased unless metastases to the liver were evident. Serum iron determinations were not done. In general, the blood picture differed in no obvious way from that of the anemia associated with chronic infection.

TABLE III. DISTRIBUTION OF MEAN RED CORPUSCULAR VALUES IN 193 CANCER PATIENTS

TYPE OF ANEMIA	NUMBER OF CASES	MCV (c.μ)			MCH (γγ)			MCHC (%)		
		ABOVE 92	NORMAL 82-92	BELOW 82	ABOVE 31	NORMAL 27-31	BELOW 27	ABOVE 36	NORMAL 32-36	BELOW 32
		%	%	%	%	%	%	%	%	%
No anemia	77	29.9	67.5	2.6	16.9	75.3	7.8	3.9	61.0	35.1
Myelopathic	65	27.7	67.7	4.6	13.9	66.1	20.0	1.5	44.6	53.9
Blood loss	33	27.3	45.4	27.3	9.1	39.4	51.5	0	21.2	78.8
Myelopathic and blood loss	15	26.7	60.0	13.3	0	53.3	46.7	0	26.7	73.3

ERYTHROPOIETIC EEFECT OF COBALTOUS CHLORIDE*

Table IV is a summary of the effects of cobaltous chloride on the sixteen patients to whom it was administered. All patients received cobaltous chloride tablets (20 mg.) by mouth in doses of 60 to 240 mg. daily. Group A includes the patients in whom there was evidence of osseous metastases and is comprised of five cases of carcinoma of the prostate and three cases of carcinoma of the breast. Two patients of this group (Cases 7 and 8) were unable to tolerate the cobalt after four and five days. This was considered too brief a period for adequate evaluation of therapy. In all other patients a significant increase in reticulocytes occurred within five to eleven days after cobalt therapy was instituted. Two patients (Cases 4 and 5) had a reticulocyte response, but death occurred before an elevation of hemoglobin might have been expected. In one patient (Case 6) the red cell count and hemoglobin remained at the initial levels for twenty-four days in spite of an adequate reticulocyte response to treatment. In the remaining three patients (Cases 1, 2, and 3), all of whom had previously failed to respond to prolonged oral therapy with ferrous sulfate, 2 Gm., and liquid extract of liver, U.S.P.—Valentine†, 1 U.S.P. unit daily, the reticulocyte response was followed by an increase in hemoglobin, hematocrit, and red blood cell levels.

Group B includes the patients in whom there was no evidence of osseous metastases and is made up of patients with various types of carcinoma as noted in Table IV. All had evidence of regional and/or distant, but not osseous, metastases. Toxic manifestations from cobalt required cessation of the drug after five or six days in four patients in this group (Cases 12, 14, 15, and 16) and precluded adequate evaluation of the therapy. In one patient (Case 13) no hematologic improvement was achieved with either iron and liver or cobalt. The remaining three patients (Cases 9, 10, and 11), one of whom (Case 9) had received previous treatment with iron and liver without effect, all had definite reticulocyte responses followed by increases in red blood count, hemoglobin, and hematocrit.

Of the sixteen patients, nine showed evidence of toxicity due to cobalt which required that the drug be discontinued in six. Toxic symptoms were pre-

*The cobaltous chloride was provided by Smith, Kline & French Laboratories, Philadelphia, Pa.

†The liquid extract of liver was provided by the Valentine's Meat Juice Co., Richmond, Va.

dominantly related to the upper gastrointestinal tract and included anorexia, nausea, epigastric burning, and vomiting. Muscle pains occurred in two patients, and in one there was an episode of substernal pain suggesting angina pectoris.

ILLUSTRATIVE CASES

Case 3. Reticulocyte and Hemoglobin Response to Cobalt.—This patient (Fig. 1) was a 73-year-old man who had a carcinoma of the prostate with x-ray evidence of metastases to the pelvis. Blood studies on admission revealed a red cell count of 3.17 million per cubic millimeter, a hemoglobin of 72 per cent, and a hematocrit of 32 per cent. The reticulocyte count was 38,040 per cubic millimeter. There was no response to the oral administration of ferrous sulfate, 2.0 Gm. a day, for twelve days, nor thereafter to liquid

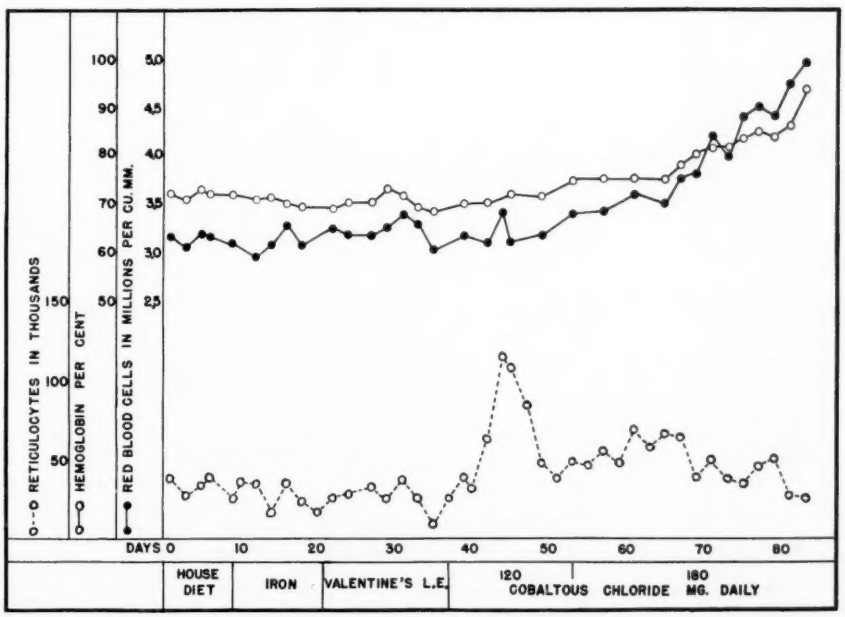

O--O RETICULOCYTES IN THOUSANDS
HEMOGLOBIN PER CENT
RED BLOOD CELLS IN MILLIONS PER CU. MM.
DAYS 0 10 20 30 40 50 60 70 80
HOUSE DIET
IRON
VALENTINE'S L.E.
120 COBALTOUS CHLORIDE 180 MG. DAILY

Fig. 1.—(Case 3.) Carcinoma of prostate with osseous metastases to pelvis. Response to orally administered cobaltous chloride after failure to respond to ferrous sulphate and liquid extract of liver, U.S.P.—Valentine.

extract of liver, U.S.P.—Valentine, 1 U.S.P. unit a day for sixteen days. There was, however, a striking reticulocyte response after cobaltous chloride, 120 mg. a day, with a peak of 115,700 per cubic millimeter on the eighth day of cobalt therapy. When the dose of cobalt was increased to 180 mg. a day, a slight reticulocytosis persisted, although there was no second peak. The red cells, hemoglobin, and hematocrit reached normal values forty-seven days after the institution of cobalt therapy.

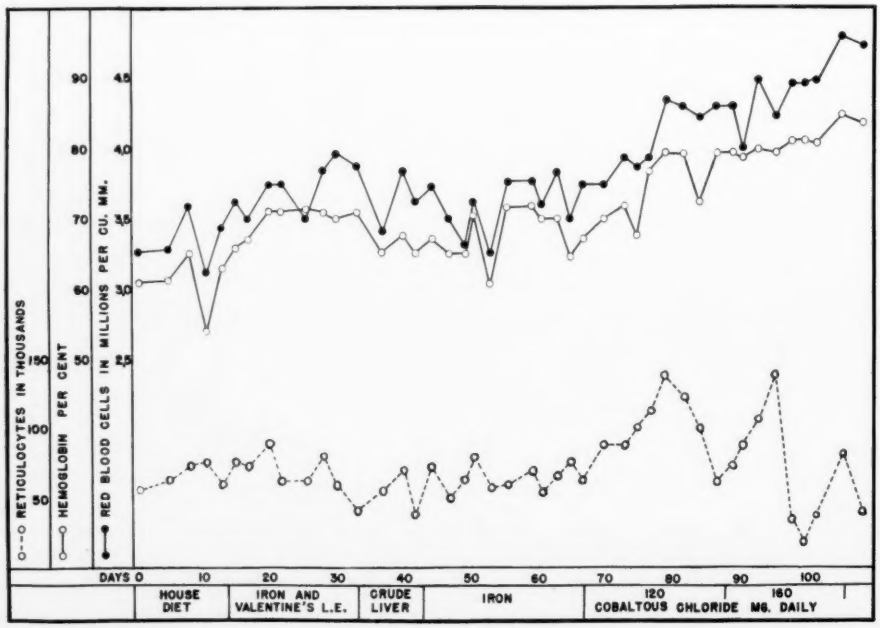
Case 2. Reticulocyte and Hemoglobin Response to Cobalt in Spite of Progression of Osseous Metastases.—This patient (Fig. 2) was a 70-year-old woman who had carcinoma of the breast with extensive metastases to the skull, spine, ribs, and pelvis (Fig. 3). The red blood cell count on admission was 3.34 million per cubic millimeter, hemoglobin 62.2 per cent, hematocrit 30 per cent, and reticulocyte count 60,800 per cubic millimeter. After a control period of thirteen days, during which there was no change in the patient's blood values, she was given a fifteen-day course of ferrous sulfate, 2.0 Gm., and liquid extract of liver, U.S.P.—

TABLE IV. THE ERYTHROPOIETIC AND TOXIC EFFECTS OF ORALLY ADMINISTERED COBALTOUS CHLORIDE IN PATIENTS WITH ADVANCED CANCER

DIAGNOSIS	CONTROL PERIOD					COBALTOUS CHLORIDE THERAPY					
	DAYS OF CONTROL	HEMOGLOBIN (%)		RETICULOCYTES (THOUSANDS)		DAYS OF THERAPY	HEMOGLOBIN (%)		RETICULOCYTES (THOUSANDS)		TOXIC EFFECTS
		INITIAL	FINAL	INITIAL	MAXIMUM		INITIAL	FINAL	INITIAL	PEAK	
Group A. Patients with osseous metastases											
Case 1. Carcinoma of breast*	42	71.0	62.9	27.9	33.1	72	72.3	92.5	41.8	89.4—5th day at 180 mg. 80.8—6th day at 240 mg.	
2. Carcinoma of breast*	14	62.2	62.0	60.8	84.0	42	68.4	84.9	61.0	143.8—11th day at 120 mg. 137.5—8th day at 160 mg.	Anorexia, muscle pains, substernal pain suggesting angina pectoris
3. Carcinoma of prostate*	6	72.0	72.5	38.0	38.4	48	69.0	92.0	24.9	115.7—8th day at 120 mg.	
4. Carcinoma of prostate	8	53.6	52.1	34.2	50.7	5	52.7	53.8	28.8	134.4—5th day at 120 mg.	
5. Carcinoma of prostate	19	48.0	40.0	31.5	31.5	14	40.0	43.0	29.1	104.4—9th day at 180 mg.	
6. Carcinoma of breast	55	58.3	44.5	23.3	61.5	24	44.3	42.4	88.7	141.1—8th day at 120 mg.	
7. Carcinoma of prostate†											Nausea and vomiting
8. Carcinoma of prostate†											Nausea, vomiting, and muscle pains
Group B. Patients without osseous metastases											
Case 9. Carcinoma of rectum with metastases to inguinal lymph nodes and with ascites*	38	67.3	66.6	9.1	60.1	65	64.9	90.0	35.8	102.0—10th day at 60 mg. 136.0—9th day at 120 mg.	Anorexia, nausea, and vomiting
10. Carcinoma of right Fallopian tube with metastases to lymph nodes and brain	11	64.8	60.4	44.5	55.5	40	60.4	73.4	43.9	112.5—8th day at 120 mg.	

11. Carcinoma of cervix	12	76.5	69.6	51.2	66.9	16	72.8	90.0	67.0	187.2—10th day at 120 mg.	Anorexia and nausea
12. Carcinoma of cervix with uremia†											Severe nausea and vomiting
13. Carcinoma of cecum*	27	44.0	46.5	26.3	66.2	14	44.3	46.0	24.7	No peak 120 mg. daily	
14. Carcinoma of breast†											Nausea and vomiting
15. Carcinoma of breast†											Nausea and vomiting
16. Carcinoma of esophagus†											Anorexia, nausea, vomiting, and burning sensation

*No response to iron and crude liver therapy.
†Drug discontinued because of toxicity before adequate period of therapy orally.


RETICULOCYTES IN THOUSANDS
HEMOGLOBIN PER CENT
RED BLOOD CELLS IN MILLIONS PER CU. MM.
DAYS 0 10 20 30 40 50 60 70 80 90 100
HOUSE DIET
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CRUDE LIVER
IRON
120 COBALTOUS CHLORIDE
160 MG. DAILY

Fig. 2.—(Case 2.) Carcinoma of breast with extensive bony metastases. Response to orally administered cobaltous chloride after failure to respond to ferrous sulfate and liquid extract of liver, U.S.P. (Valentine), or crude liver powder or ferrous sulfate alone.

Fig. 3.—(Case 2.) X-ray of pelvis before therapy showing osteolytic lesions.

Valentine, 1 U.S.P. unit daily by mouth. This was followed by an eight-day course of crude liver powder, 40 Gm. daily, and then by a nineteen-day course of ferrous sulfate, 2.0 Gm. a day. There was no change in the blood values at the end of this time and the reticulocyte counts showed no significant increase. The patient was then given cobaltous chloride, 120 mg. daily by mouth. On the eleventh day the reticulocyte count reached a peak of 143,800 per cubic millimeter. On the eighteenth day the dose was increased to 160 mg.

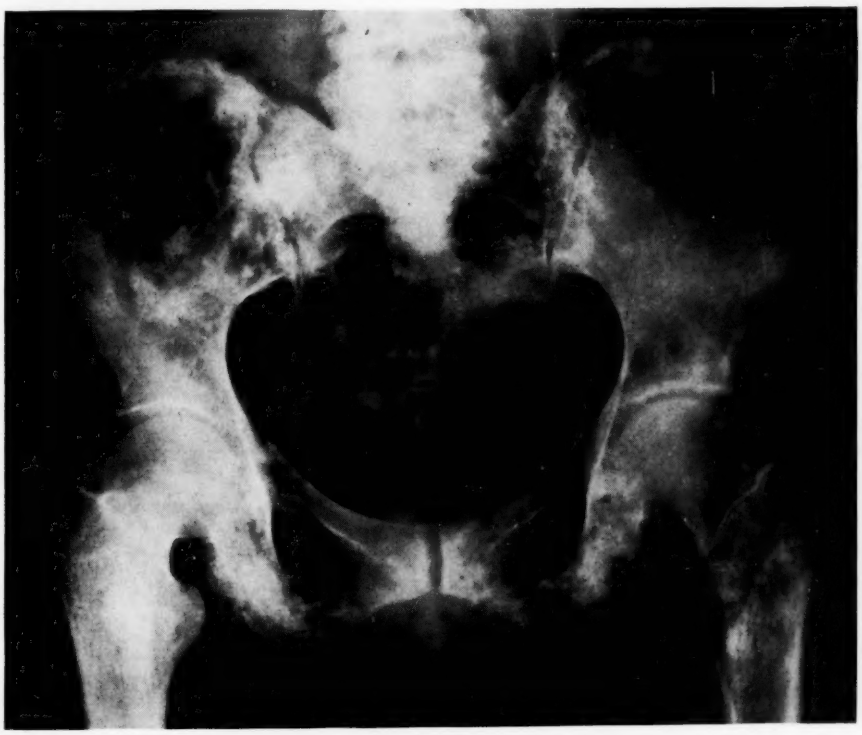

Fig. 4.—(Case 2.) X-ray of pelvis after cobaltous chloride therapy showing progressive osteolytic changes.

a day and eight days thereafter another reticulocyte peak of 137,500 per cubic millimeter occurred. Following the reticulocyte response, the red cells, hemoglobin, and hematocrit rose steadily and reached normal levels by the end of three weeks of cobalt therapy. An x-ray of the pelvis, taken at the conclusion of the cobalt therapy, showed that destruction of bone had progressed (Fig. 4).

Case 5. Reticulocyte Response to Cobalt, With Death Occurring Before Hemoglobin Response Might Have Been Expected.—This patient (Fig. 5) was a 75-year-old man with carcinoma of the prostate who was admitted for terminal care. X-rays revealed that the entire skeleton was extensively involved with osteoblastic metastases. Admission studies showed a red cell count of 2.42 million per cubic millimeter, hemoglobin 48 per cent, hematocrit 23 per cent, and reticulocytes 31,500 per cubic millimeter. During a control period of nineteen days on the usual hospital diet, there was no change in the red cell or hemoglobin levels or in the reticulocytes. On the twentieth day after admission the patient began to receive cobaltous chloride, 120 mg. a day, by mouth. He tolerated the drug very well, and after six days the dose was increased to 180 mg. a day. There was a prompt and striking increase in reticulocytes, but the patient expired suddenly fourteen days after the institution of cobalt therapy. On the day of death the reticulocyte count was 104,400 per cubic millimeter. There was no significant increase in red cells, hemoglobin, or hematocrit.

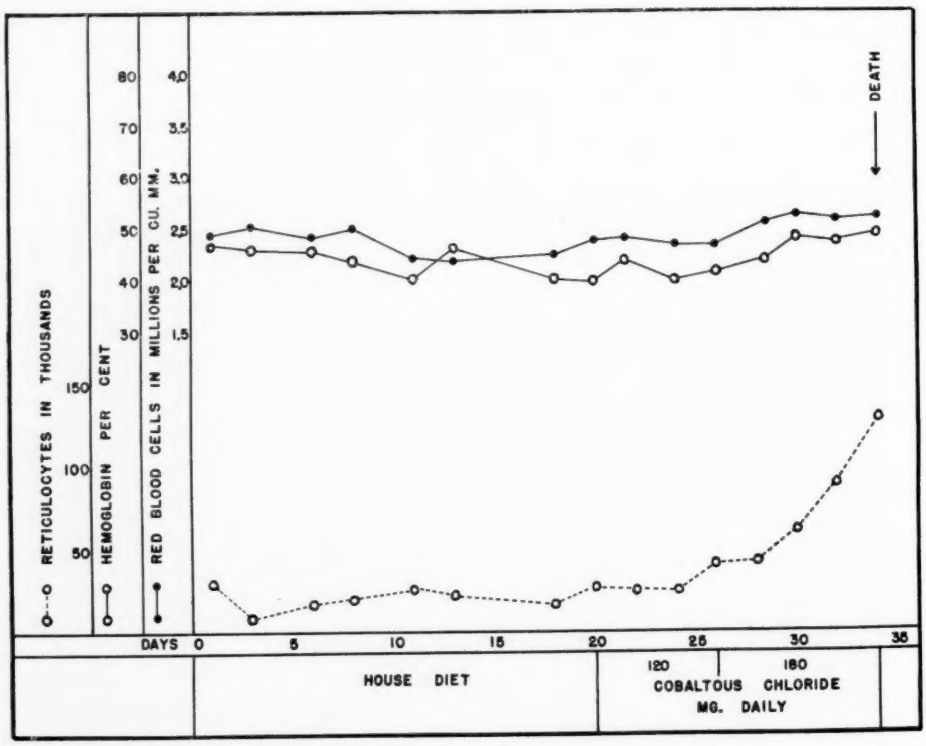

Fig. 5.—(Case 5.) Carcinoma of prostate with extensive osteoblastic metastases. Reticulocyte response to orally administered cobaltous chloride.

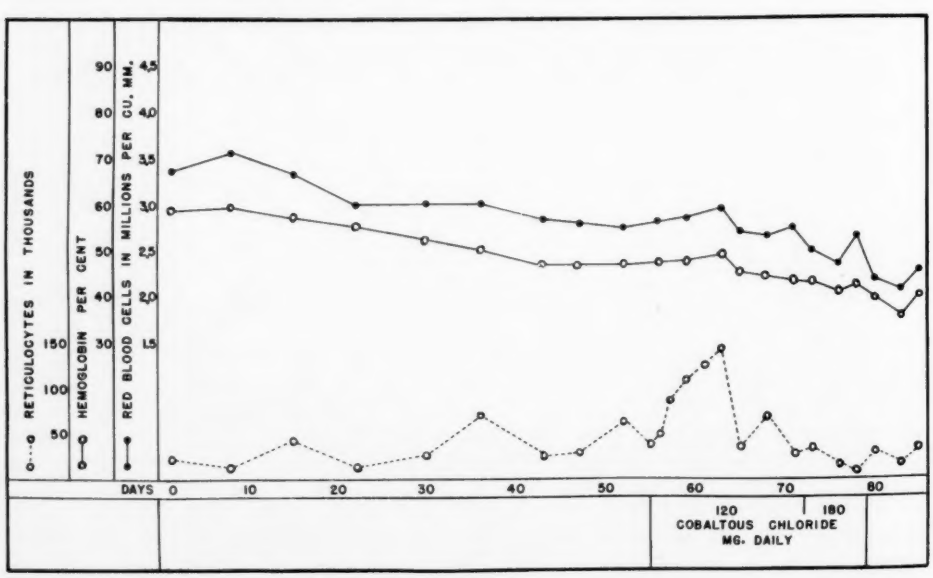

Fig. 6.—(Case 6.) Carcinoma of breast with extensive metastases to bone. Reticulocyte response with no hemoglobin or red cell response to orally administered cobaltous chloride.

Case 6. Reticulocyte Response to Cobalt Not Followed by Hemoglobin Response in Spite of Adequate Therapy.—This patient (Fig. 6), a 58-year-old woman, was admitted for metastatic carcinoma originating in the left breast. She complained of back pain, and x-rays demonstrated extensive skeletal lesions of the metastatic type with a fracture of the left sixth rib and compression of the eleventh thoracic and third lumbar vertebrae. The red cell count was 3.3 million per cubic millimeter, hemoglobin 58.3 per cent, hematocrit 28.8 per cent, and reticulocyte count 23,300 per cubic millimeter. During the control period of fifty days,

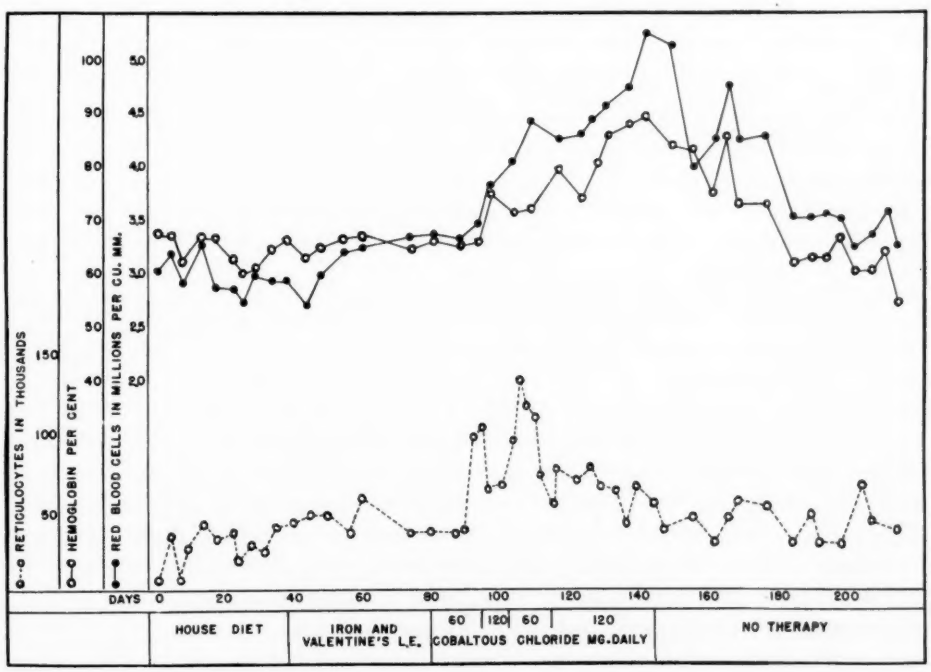

Fig. 7.—(Case 9.) Carcinoma of rectum with metastases to lymph nodes but no evidence of bony metastases. Response to orally administered cobaltous chloride after failure to respond to ferrous sulfate and liquid extract of liver, U.S.P.—Valentine. Hemoglobin and red blood cell levels returned to previous levels two months after cobaltous chloride was discontinued.

while the patient was an outpatient and received no specific therapy, there was a slight decline in the red cell count, hemoglobin, and hematocrit. She was then admitted to the hospital and was given cobaltous chloride, 120 mg. daily. There was a prompt reticulocyte response with a peak of 141,100 per cubic millimeter on the eighth day. The reticulocyte response was not followed by a rise in the red cells and hemoglobin, and, in fact, these values continued to decline slowly. There was no evidence of blood loss. On the seventeenth day after cobalt was begun, the dose was increased to 180 mg. a day. Thereupon the patient developed nausea, vomiting, and muscle pains; and after eight days on this dosage the drug was discontinued. No reticulocyte response was noted and the red cells, hemoglobin, and hematocrit continued their slow decline.

Case 9. Reticulocyte and Hemoglobin Response to Cobalt With Return of Blood Values to Pretreatment Levels After Cessation of Therapy.—This patient (Fig. 7) was a 70-year-old woman who had had an abdominoperineal resection for a carcinoma of the rectum two years before admission. Following the operation, however, she gradually developed ascites and enlargement of the inguinal lymph nodes. Repeated stool examinations were negative for

blood. Skeletal x-rays showed no evidence of metastases and aspiration of the iliac marrow revealed no tumor cells. On admission the red cell count was 3.04 million per cubic millimeter, hemoglobin 67.3 per cent, hematocrit 29.2 per cent, and reticulocyte count 9,100 per cubic millimeter. During the control period of thirty-eight days on the usual hospital diet, there was no change in the red cells, hemoglobin, or hematocrit. The reticulocytes varied between 9,100 and 61,100 per cubic millimeter. The patient was then given ferrous sulfate, 2.0 Gm., and liquid extract of liver, U.S.P.—Valentine, 1 U.S.P. unit daily by mouth, for forty-two days. With this therapy there was no significant change in red blood count or hemoglobin, and the highest reticulocyte count was 59,000 per cubic millimeter. The patient was then given cobaltous chloride, 60 mg. a day. On the fourteenth day the reticulocytes reached a peak of 102,000 per cubic millimeter. On the following day the dose of cobalt was doubled. Because of gastrointestinal symptoms, after eight days the dose was reduced to 60 mg. a day. Nevertheless, a second reticulocyte peak of 136,000 per cubic millimeter occurred eleven days after the doubled dose had been started. Because the patient tolerated 60 mg. of cobaltous chloride a day for thirteen days, the dose was again doubled and the patient was successfully maintained for thirty days on 120 mg. a day. There was no further reticulocyte peak. During the period of cobalt administration, however, the red cell count, hemoglobin, and hematocrit reached normal levels. When the drug was discontinued after a total of sixty-five days because of a recurrence of the gastrointestinal symptoms, the red cells, hemoglobin, and hematocrit returned gradually to their pretreatment levels over a period of about two months.

DISCUSSION

Cobaltous chloride or cobaltous nitrate has been shown to produce polycythemia in normal mammals, Amphibia, and birds. Weissbecker and Maurer[13] and Berk, Burchenal, and Castle[12] have reported that the daily oral administration of cobaltous chloride to normal subjects regularly produces reticulocyte responses followed by increases in red cell, hemoglobin, and hematocrit values. Wintrobe and others[14] have shown that in rats cobalt will eliminate the anemia previously produced by injections of turpentine. The work of Berk and associates[12] and of Robinson, Watson, and Kark[15] indicates that the drug given orally to patients with the anemia of chronic infection produces a mild or moderate increase in reticulocytes within ten days, followed by a rise of the red blood count and hemoglobin to normal levels. Subsequently Kark[16] demonstrated hematologic responses to cobalt in patients with anemia associated with prostatic carcinoma. Berk and co-workers[12] showed that patients with refractory anemia fail to respond to cobalt therapy and suggested that, in general, significantly anemic patients of any variety could not be expected to be benefited.

The resemblance of anemia due to chronic infection and the myelopathic anemia of cancer patients is striking in many respects. (1) Both anemias are slight or moderate in degree; that is, the red blood cell count is usually over three million per cubic millimeter and the hemoglobin level about 60 per cent. (2) Both anemias are normocytic or slightly microcytic and normochromic or slightly hypochromic. (3) Both anemias show normal or very slightly increased reticulocyte counts and normal plasma bilirubin values. (4) Both anemias fail to respond to other known hematopoietic agents such as iron or liver. (5) Brock and Hunter[17, 18] have shown that massive doses of iron are not fully

utilized. Wintrobe and others[14, 19] demonstrated that hypoferremia occurs in anemia associated with chronic infection and pointed out the accumulation of iron in the liver, spleen, and tissues which occurs in cases of anemia associated with malignancy. These observations suggest that in both anemias the tissues have an increased affinity for iron, presumably due to a fault in the intermediary metabolism of iron utilization. (6) Clinically, the similarity between these two anemias is further suggested by the frequent occurrence in cancer patients of necrosis of neoplastic tissue which is often associated with secondary infection and inflammation.

In view of the evidence that cobalt is effective in the anemia associated with infection and in view of the similarities between the anemia associated with cancer and that associated with infection, it seemed logical to try cobaltous chloride as a hematopoietic agent for these cancer patients. A reticulocyte response after administration of the drug was observed in all six patients with metastases to bone and in three of four patients without metastases who could tolerate cobalt for an adequate trial. These observations offer further evidence for the possible identity of the defect producing anemia in cancer and in chronic infection.

Although the mechanism of action of cobalt on erythropoiesis is as yet not completely understood, it has been suggested by numerous authors[20] that cobalt inhibits respiration in animal tissue and tumors, thus producing cellular "anoxia" in the bone marrow and in turn inducing a compensatory polycythemia. Recent work[21] has demonstrated that cobalt may induce the formation of reversible complexes of the amino acids, histidine and cysteine, with oxygen. It is possible, therefore, that cobalt might combine with and block sulfhydryl groups and perhaps other groups active in cellular respiration, and thus interfere with the transport of oxygen to the erythroid cells of the bone marrow. There is no in vivo evidence that the presumed anoxia is due to the formation of methemoglobin, although ascorbic acid, an agent which effectively reduces methemoglobin, inhibits the effects of cobalt in producing polycythemia in rabbits.[22, 23] It also has been postulated that the hematopoietic effect of cobalt is concerned with its action on the bacterial flora of the gut in promoting the synthesis of vitamin B_{12}.[24-28] This is unlikely, however, since cobalt does not appear to be effective in pernicious anemia in relapse,[12] nor is liver extract containing vitamin B_{12} effective in the treatment of the mild anemias that respond readily to cobalt.

Whatever the mode of action of cobalt on erythropoiesis may be, the hematologic responses of the patients in this study as contrasted with the complete ineffectiveness of cobalt in refractory anemia strongly suggest that the erythropoietic reserve of the bone marrow in these patients had not been totally exhausted by neoplastic replacement.

That marrow replacement is not usually an important factor in the etiology of anemia associated with cancer is substantiated by the results of the statistical analysis of our cases, which indicates that there is no positive correlation between the incidence of anemia and the occurrence of osseous metastases. As

shown in Tables I and II it may be seen that the patients with anemia without osseous metastases actually outnumber those with metastases to bone; furthermore, this relationship exists even in that group designated as due to myelopathy alone. Conversely, anemia is equally frequent in patients with metastases and in those without metastases.

It would thus appear that the etiology of myelopathic anemia in cancer patients and that of the secondary anemia of chronic infection are related. Both anemias may be due either to inhibition of the bone marrow by toxic products elaborated by tumor tissue or by the infectious process or to a disturbance of the metabolism of certain amino acids which are required to combine with iron for the production of the hemoglobin. It does not seem likely that neoplastic tissue removes some hematopoietic factor as an essential for its autonomous growth.[5]

Finally, it should be noted that in all the patients treated with cobalt, with or without hematologic response, clinical improvement was not otherwise apparent. Indeed, the patients' appetites were usually impaired, they felt weaker, and they acted more mentally depressed. Substernal pain suggesting angina pectoris developed in one patient during cobalt therapy. This is in striking contrast to the general clinical improvement which accompanies hematologic improvement in other types of anemia, such as in pernicious anemia when treated with vitamin B_{12} or liver or in iron deficiency anemia when treated with iron salts. It should be emphasized that cobalt therapy did not alter or arrest the primary disease. Although under cobalt therapy some of the patients maintained high hemoglobin and red cell levels, the underlying disease continued to progress and eventually all died of cancer.

SUMMARY

Morphologic studies of the peripheral blood and bone marrow were made in 193 patients with advanced cancer. One hundred and sixteen (60.1 per cent) were considered to be anemic because the hemoglobin level was 80 per cent or below. Of these 116 anemic patients, the hemoglobin was between 70 and 80 per cent in fifty-two, between 55 and 69 per cent in forty-two, and between 38 and 54 per cent in twenty-two.

When the anemias were classified according to pathogenesis, 28.5 per cent were due to blood loss alone, 2.6 per cent to hemolysis, 56 per cent were of the myelopathic type, and 12.9 per cent of the myelopathic type complicated by blood loss.

Of the 193 patients, fifty (25.9 per cent) had evidence of osseous metastases. Of those with such metastases, twenty-four (48 per cent) were anemic; twenty-six (52 per cent) were not anemic.

In sixteen anemic patients (eight with and eight without metastases to bone) cobaltous chloride, 60 to 240 mg. daily, was employed to ascertain whether the bone marrows of these patients were capable of responding to such a hematopoietic stimulus. In all six patients with osseous metastases who received an adequate trial on the drug, the administration of cobalt was followed by reticulo-

cyte responses. Two of these patients died before a rise in hemoglobin might have been expected. One patient failed to show any rise in hemoglobin, although the cobalt was continued for twenty-four days. In the other three patients of this group, the reticulocytosis was followed by elevations to normal of the red cell counts, hemoglobin, and hematocrit levels. Of the four patients without metastases to bone who could tolerate cobalt for an adequate period, three showed definite reticulocyte responses followed by increase in red cells, hemoglobins, and hematocrits. The other patient showed no reticulocyte or other hematologic response to iron, liver, or cobalt therapy.

These results indicate that the presence of metastases to bone usually has no relation to the development of anemia in cancer patients, and that cobalt therapy in cancer patients with anemia may increase erythropoiesis regardless of metastases to bone. Cobalt therapy fails to produce subjective improvement and often causes symptoms referable to the gastrointestinal tract and in some instances suggestive of angina pectoris.

From these results it is concluded that anemia associated with cancer is not usually due to bone marrow replacement by neoplastic tissue.

We are indebted to Dr. William B. Castle and Dr. Allyn B. Ley for many stimulating suggestions and advice in preparing this report.

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BLOOD ERGOTHIONEINE LEVELS IN DISEASE

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IN AN earlier report[1] the mean blood ergothioneine level in patients with diabetes mellitus was shown to be significantly higher than the mean value for a comparable group of normal subjects. A number of other diseases have now been investigated in an effort to discover a common factor which may be associated with changes in blood ergothioneine levels. It is hoped that this survey may indicate some conditions in which a more exhaustive study should be made, and stimulate investigation into the physiology of this interesting erythrocyte constituent.

METHODS AND MATERIALS

Ergothioneine values were determined by the diazo method described by Hunter[2] and the results are expressed in milligrams per 100 ml. corpuscles. A correction was made, as described in our previous paper,[1] for the colorimetric value contributed by plasma.

Further investigation into the factors connected with the development of a bronze rather than the expected violet-pink color of the final solution in this test did not enable us to identify the interfering substance. This substance appeared to originate from the plasma. When four precautions were observed, the frequency of appearance of the bronze color was much reduced. (1) Blood was used as soon as possible after venepuncture. (2) The sample was refrigerated during storage. (3) Hemolysis was avoided. (4) The oxalate concentration was not greater than 2 mg. per milliliter of blood.

In most samples in which an interfering color was found, it was possible to obtain a satisfactory determination by the following procedure.

(a) The plasma was pipetted off.

(b) One per cent saline was added to the corpuscles and the resulting suspension was mixed and centrifuged.

This was repeated four times.

(c) The cells, suspended in one per cent saline solution in approximately a 50 per cent concentration, were then carried through the test in the usual manner. It was found that precipitation was not satisfactory unless 0.0020N acetic acid was substituted for the usual 0.0030N acid. Final values per 100 ml. corpuscles were calculated by determining the hematocrit readings of each saline suspension of cells. No loss of ergothioneine occurred during this procedure.

Sources of Blood.—The data from the same group of ninety-four normal subjects which were reported earlier[1] were once again used for comparison and are given in Tables I and III.

Blood from patients with tuberculosis was obtained from Indian and Eskimo patients at the Charles Camsell Indian Hospital, and from white patients at the Provincial Tuberculosis Wards in two city hospitals.

All other estimations were made on samples which had been taken for other blood chemistry work at two city hospitals.

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RESULTS

Part I. Tuberculosis.—Blood ergothioneine levels were determined on sixty-five Indian and four Eskimo patients suffering from tuberculosis. The mean ergothioneine value for sixty-nine patients was 18.0 mg. per cent, a level well above the normal mean of 9.6 mg. per cent. (Table I.) This series was then divided into smaller groups according to the type of tuberculosis and the clinical degree of infection.

It was interesting to note the gradual decrease in mean ergothioneine values which corresponds to the increase in severity of tuberculosis. Two patients who subsequently died had levels of 7.6 and 19.0 mg. per cent, and three others with far-advanced disease showed relatively low levels of 6.7, 6.9, and 11.5 mg. per cent. Table I shows that the mean ergothioneine value for far-advanced tuberculosis was approximately 5 mg. per cent lower than that for controlled or minimal disease.

Single cases each of renal tuberculosis and tuberculoma exhibited extraordinarily high values.

Three Indian patients, who were admitted as suspected cases of tuberculosis, were subsequently found to be free of the disease. One other Indian child was being treated for a congenital dislocation of the hip. These four patients (none is included in Table I) showed the surprisingly high values of 14.5, 23.6, 22.1, and 42.6 mg. per cent respectively. The first three patients suffered from lung infections other than tuberculosis, but the 3-year-old boy with the congenital hip dislocation apparently otherwise was well.

TABLE I. BLOOD ERGOTHIONEINE LEVELS OF 65 INDIAN AND 4 ESKIMO PATIENTS WITH TUBERCULOSIS

	NUMBER OF PATIENTS	MEAN AGE (YR.)	MEAN ERGOTHIONEINE (MG. PER 100 ML. CORPUSCLES)
Normal subjects	94	29.5	9.6
All patients with tuberculosis	69	18.0	18.0*
Controlled or minimal pulmonary tuberculosis	24	15.6	19.1
Moderately advanced pulmonary tuberculosis	18	20.0	16.8
Far-advanced pulmonary tuberculosis	15	23.8	14.2
Tuberculosis of bone	10	12.8	14.8
Tuberculoma	1	8	48.2
Renal tuberculosis	1	15	52.3

t value at 1 per cent level = 2.609.
*Range 4.3 to 53.0 mg. per cent. Standard error of the mean, 1.5. t value found = 3.674.

It was possible that peculiarities of Indian diet under home conditions might have influenced the ergothioneine level. On inquiry it was found that many of the patients showing abnormally high ergothioneine values had been hospitalized, and on routine hospital diets, for as long as three years.. In confirmation of the belief that the Indian diet was not an important factor, it was also found that many of the recently admitted patients showed values within normal limits.

Forty-seven white patients with tuberculosis showed a mean ergothioneine level of 10.9 mg. per cent. (Table II.) This was not significantly higher than the normal value of 9.6 mg. per cent. When white tuberculous patients were separated into those with pulmonary and those with extrapulmonary infection, a mean ergothioneine level significantly higher than normal was found in the latter group, which consisted mainly of patients with tuberculous disease of bone.

Because of inadequate data, no attempt was made to classify the cases according to severity of tuberculous disease in the white population.

TABLE II. BLOOD ERGOTHIONEINE LEVELS IN TUBERCULOUS WHITE PATIENTS

	NUMBER OF CASES	MEAN AGE (YR.)	MEAN ERGOTHIONEINE (MG. %)	STANDARD ERROR OF MEAN	t VALUE
Pulmonary tuberculosis	38	34.7	9.9	0.71	--
Nonpulmonary tuberculosis	9	--	15.2	1.96	4.697*
Combined	47	44.9	10.9	0.74	1.878

*t value at 1 per cent level = 2.626.

Part II. Other Diseases.—Small numbers of patients with a variety of diseases were investigated. To facilitate analysis, many of these were grouped under broader classifications, confined as much as possible to related conditions. The results are presented in ascending order of mean ergothioneine values in Table III. The author reviewed the clinical history and laboratory findings and concurred with the diagnoses in all cases.

The mean ergothioneine values for the following diseases showed no significant variation from the normal, and are not included in Table III: hernias and hydroceles (thirteen cases); pregnancy, normal and toxic (eight); carcinomas of the prostate, breast, bowel, stomach, bone, bladder, brain, uterus, cervix, pancreas (twenty-eight); arthritis, rheumatoid and osteoarthritis (forty-one); goiters, nontoxic and toxic (eleven); abortions (thirteen); leucemias, acute and chronic myelogenous (four), and chronic lymphatic (one); multiple myeloma (one); lupus erythematosus (one).

The object of this investigation was to accumulate mean ergothioneine values from as representative a group of diseases as possible. It was hoped that in this way potential avenues for further investigation might become apparent, but it is also obvious that no conclusions can be reached from the results of such a small series. With these reservations, the following observations are made on some of the more significant data presented in Table III.

Only one disease group, hypothyroidism, showed a significant decrease in the ergothioneine mean.

In cases of both appendicitis and nose and throat infection there was a significant increase in mean ergothioneine level in the acute cases, but there were close to normal values in the chronic.

TABLE III. A COMPARISON OF THE MEAN BLOOD ERGOTHIONEINE VALUES FOUND IN VARIOUS DISEASES WITH THE MEAN VALUE OF NORMAL SUBJECTS

DISEASE	NUMBER OF PATIENTS	MEAN AGE (YR.)	MEAN ERGOTHIONEINE (MG. %)	STANDARD ERROR OF THE MEAN	t VALUE CALCULATED
Normal subjects	94	29.5	9.6	0.32	
Appendicitis					
1. Acute	7	25.2	12.7	1.87	3.516§
2. Chronic	10	22.5	8.2	--	
Combined	17	23.5	10.1	1.12	0.562
Peptic ulcers					
1. Perforated	3	33.5	4.3	--	
2. Hemorrhaging	2	47.0	15.6	--	
3. Uncomplicated	8	53.1	11.4	--	
Combined	13	48.8	10.4	1.41	0.795‡
Lung infections					
1. Lobar pneumonia	9	38.6	10.2	--	
2. Bronchopneumonia	11	52.6	11.4	--	
3. "Pneumonitis" virus pneumonia	15	35.2	10.3	--	
4. Bronchiectasis and lung abscesses	5	51.2	13.0	--	
Combined	40	--	10.9	0.75	1.876†
5. Chronic bronchitis	5	52.2	13.0	--	
6. Asthma	3	48.3	9.4	--	
Combined	8	--	11.7	1.90	1.717‡
All lung diseases combined	48	44.8	11.1	0.69	2.246*
Diabetes with associated diseases (hypertension, etc.)	19	57.3	11.9	1.68	2.229‡
Anemias					
1. Hypochromic and hypoplastic	6	52.4	13.2	1.95	2.655‡
2. Pernicious	2	46.5	4.1	--	--
Combined	8	50.7	10.9	2.09	1.044‡
Nose and throat infections					
1. Acute	12	31.0	12.9	1.75	2.045‡
2. Chronic or recurrent	9	24.6	8.6	--	
Combined	21	27.5	11.0	1.10	1.640†
Gynecological diseases					
1. Endometrial hyperplasia	8	42.7	10.0	--	
2. Pelvic inflammatory disease	7	39.6	12.6	--	
3. Cystoceles and fibroids	7	42.6	11.6	--	
Combined	22	41.6	11.3	0.81	2.214†
Gall bladder disease					
1. Cholecystitis and cholelithiasis	10	53.3	12.1	1.66	2.240‡
2. Cholecystitis	3	46.5	9.2	--	--
Combined	13	52.2	11.4	1.42	1.788‡
Infections other than respiratory					
1. Cellulitis	13	36.9	9.4	--	--
2. Infectious arthritis and osteomyelitis	4	24.5	17.6	--	--
3. Abscesses	6	33.6	12.0	--	--
Combined	23	33.0	11.6	1.19	2.290†
Muscle and joint injury					
1. Muscle injuries	14	34.1	11.5	--	--
2. Protruded intervertebral discs	9	40.7	12.1	--	--
Combined	23	36.7	11.7	1.29	2.315†
Coronary and hypertensive vascular disease					
1. Hypertension	15	68.6	12.1	--	--
2. Cerebral vascular accident	6	71.0	13.1	--	--
3. Coronary thrombosis	11	60.5	13.3	--	--
Combined	32	66.3	12.7	1.39	3.271†

(Continued on opposite page.)

TABLE III—CONT'D

DISEASE	NUMBER OF PATIENTS	MEAN AGE (YR.)	MEAN ERGOTHIONEINE (MG. %)	STANDARD ERROR OF THE MEAN	t VALUE CALCULATED
Urinary tract disease					
1. Nephritis	4	36.2	16.3	--	--
2. Nephrosis	3	5.5	21.0	--	--
Combined	7	--	18.3	2.78	6.017‡
3. Prostatic obstruction	7	70.7	10.2	--	--
4. Miscellaneous	7	50.6	10.3	--	--
Urinary tract disease combined	21	49.2	12.9	1.30	2.189†
Dermatitis	6	52	13.6	2.92	2.764‡
Fractures					
1. Old	5	41.0	15.2	--	--
2. Recent	9	39.0	13.2	--	--
Combined	14	39.1	13.9	1.86	3.946‡

t value at	1 per cent level:	5 per cent level
*2.609		1.976
†2.616		1.979
‡2.626		1.984
§2.632		1.987

There was a marked contrast between the values found for hypochromic and pernicious anemias. Both of the latter patients were suffering from exacerbations of their disease and had erythrocyte counts of between 1 and 2 million.

There were thirteen cases of cholecystitis. In ten of these there were associated calculi, and the mean ergothioneine level was almost 3 mg. per cent higher than the value for the three cases without stones. In only one of the cases of cholecystitis with calculi was there any increase in serum bilirubin. This was in a 78-year-old woman who also had an enlarged spleen and showed evidence of functional liver damage. Her ergothioneine level was 7.1 mg. per cent.

In the eighth group there were two cases of osteomyelitis, one case of gonococcal arthritis of the knee and one case of infective arthritis of the ankle. These are worthy of mention because the first three showed high ergothioneine values, 16.0, 19.2, and 30.7 mg. per cent respectively, whereas the last case, a 3½-year-old girl, was found to have a lower than normal value of 5.6 mg. per cent.

Increased values were found in four cases of nephritis and three of nephrosis. Two of the cases of nephrosis were in children, ages 4 and 6 years, whose cholesterol values ranged between 800 and 1,000 mg. per cent. The ergothioneine levels were 12.4 and 19.4 mg. per cent. The third patient with nephrosis was a woman who died in uremia secondary to a toxic nephrosis caused by an attempted abortion. In this case the ergothioneine level reached the high value of 31.3 mg. per cent.

In none of the cases of prostatic obstruction was there a serious degree of nitrogen retention. The mean value of 10.2 mg. per cent was close to the normal mean of 9.6 mg. per cent.

In one of the six cases classified as dermatitis the patient had an urticarial type of rash and showed an ergothioneine value of 16.4 mg. per cent. The

highest ergothioneine value in this group was found in an 81-year-old woman suffering from hypostatic dermatitis—26.3 mg. per cent.

The difficulties in assessing our findings in the presence of more than one disease are illustrated by the following example. Out of the nine cases of protruded intervertebral discs (the tenth group) there were six in which the ergothioneine values ranged between 8.8 and 10.7 mg. per cent. The remaining three values were 2.4, 19.4, and 30.5 mg. per cent. These last two values were found in two patients, one of whom was suffering from hypertension (215/135) and the other from a chronic pelvic inflammatory disease. It already has been shown that both of these conditions in themselves are associated with increased ergothioneine levels.

Certain other diseases, because they were too few to classify, made up a miscellaneous group. The more interesting are listed in Table IV, either because they represented rather uncommon conditions or because they showed abnormal ergothioneine values.

TABLE IV. BLOOD ERGOTHIONEINE VALUES IN VARIOUS DISEASES

DISEASE	AGE (YR.)	ASSOCIATED CONDITIONS	ERGOTHIONEINE (MG. %)
Syphilis, congenital	25		9.9
Syphilis, acquired	62	Hemorrhoids, hypertension	7.1
Syphilis, acquired	43	Ruptured aortic aneurysm, died same day	11.7
Portal cirrhosis	63	Chronic bronchitis, laparotomy 6 days previously	6.6
Homologous serum jaundice	38	Jaundice 1 wk. Died 6 days later. Massive hepatic necrosis. Bronchopneumonia. Repaired (perforated) duodenal ulcer 3 mo. previously	7.1
Acute pancreatitis	60	Tested after recovery. 1 mo. after admission	12.3
Adenoma of pancreas with hyperinsulinism	33	Tested 7 days postoperatively	10.0
Gout	--		10.3
Small bowel obstruction due to postappendectomy evisceration (4 weeks ago)	49	Serum albumin, 1.03 Gm.; globulin, 4.01	14.2
Bowel obstruction, due to fecal impaction	78	Osteoarthritis. Senile cataracts	15.3
Capillary hemangioma, lip	60	Chronic bronchitis	19.4
Thermal burn, arm	--		20.4
Laceration, thigh	17		3.7
Adrenal insufficiency	69	Acute respiratory infection	3.3

In a number of patients blood ergothioneine values were determined over the course of weeks. In none of these was there any significant change in value. In one patient suffering from disseminated lupus erythematosus in whom the pretreatment value was normal, there was a slight fall in ergothioneine level following the initiation of cortisone therapy. During the course of the disease the patient developed a pulmonary embolus with infarction. Coincident with this accident the blood ergothioneine value increased from 8.8 mg. per cent to 12.1 mg. per cent. Three weeks later it still ranged between 14.5 and 16.5 mg. per cent. Shortly after this the patient was discharged in a much-improved condition.

In another patient who was a barbiturate addict, blood ergothioneine values were determined hourly from 8 A.M. until 4 P.M. These values ranged from 10.3 to 11.0 mg. per cent, with the exception of the value at 10 A.M. (12.6 mg. per cent) for which no explanation is advanced.

Several other isolated but interesting findings were made in the course of the survey.

Work by another member of this Department[3] on ergothioneine levels in rats suggested that an increase in ergothioneine value occurred after rats were bled. For this reason estimations were made on students who donated blood to the Red Cross Blood Donor Clinic. These estimations were carried out before the donations and at one and two weeks after the donation. The results are tabulated in Table V.

At the end of one week ergothioneine values showed both increases and decreases from the initial levels. By the end of the second week the ergothioneine levels of all subjects were higher than their respective initial values, even though in some cases the difference was small.

In a single case simultaneous ergothioneine estimations were made on peripheral blood and bone marrow. An ergothioneine value of 10.4 mg. per cent was found in the peripheral blood, and a higher level of 14.9 mg. per cent in the bone marrow.

TABLE V. BLOOD ERGOTHIONEINE LEVELS OF SEVEN NORMAL SUBJECTS BEFORE AND AFTER BLOOD DONATIONS*

	ERGOTHIONEINE (MG. %)		
STUDENTS	BEFORE DONATIONS	1 WEEK AFTER DONATION	2 WEEKS AFTER DONATION
A	6.9	3.9	7.9
B	4.1	7.9	5.7
C	8.0	6.4	9.3
D	7.3	6.3	7.7
E	5.9	6.3	6.7
F	8.5	6.5	10.3
G	6.2	7.6	8.4

*Approximately 350 milliliters.

CONCLUSION

There are many aspects of this problem which should be explored. Comparatively few biochemical or physiologic studies have been made, and our work shows that these should be carried out. In all normal subjects who were investigated there was a range of only 14 mg. per cent, with a standard deviation of 3.1 mg. per cent. The abnormal findings in various diseases emphasize the yet unanswered question of the function of ergothioneine. It seems unlikely that there would be no role assigned to a constituent of such a vital structure as the red blood cell, especially since ergothioneine has been shown to possess a definite oxidation-reduction potential.[4]

SUMMARY

The problem of the development of an interfering color in the Hunter diazo test for blood ergothioneine was investigated and methods were suggested for avoiding and removing this effect.

The mean blood ergothioneine level of sixty-nine Indian and Eskimo tuberculous patients was found to be significantly higher than the mean ergothioneine value of normal white subjects. No ergothioneine estimations were made on a normal Indian and Eskimo population. Four of these patients with diseases other than tuberculosis showed high blood ergothioneine levels.

Thirty-eight white patients with pulmonary tuberculosis showed no significant variation in mean blood ergothioneine value. Nine white patients with extrapulmonary tuberculous lesions showed a significant increase in mean ergothioneine level.

Blood ergothioneine values were determined in 414 other patients. Significant variations in the mean erogthioneine values were found in hypothyroidism, acute appendicitis, anemia, coronary and hypertensive vascular disease, nephritis and nephrosis, dermatitis, fractures, lung infections, diabetes with associated disease (hypertension, etc.) acute infections of the nose and throat, gynecologic conditions, cholecystitis with cholelithiasis, infections other than respiratory, and muscle and joint injuries.

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THE EFFECT OF VARIOUS PROTEIN HYDROLYSATES AND AMINO ACIDS ON THE UREMIC RAT

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THE majority of investigators agree that the administration of protein-rich foods definitely shortens the survival time of rats with experimentally induced uremia. It is not likely that this toxic effect is due to urea alone, as there is little evidence that urea per se, in levels observed in complete anuria and uremia, is very toxic to the body.[1-8] Available information does not indicate clearly whether the amino acids or other constituents of protein are the cause of toxicity to the renal-insufficient organism.

In 1926 Anderson[9] reported that partially nephrectomized rabbits maintained on a high or low protein diet showed changes in the blood urea and creatinine that could be correlated with the diet. Lundin and Mark reported[10] that dogs deprived of 75 per cent of their renal tissue developed a high blood nonprotein nitrogen when kept on a high protein diet which rapidly returned to normal when they were maintained on a low protein diet. Chanutin, Ferris, and Wood[11] found that removal of 80 to 85 per cent of the renal tissue of white rats resulted in cardiac hypertrophy and hypertension when the animals were maintained on a high protein diet but not when urea or creatine were added to the diet to provide comparable amounts of nitrogen.

Blatherwick and co-workers[12] found that unilaterally nephrectomized rats developed nephritis more frequently on a high protein diet than did similar animals on the control ration. Kirk[13] demonstrated a greater and more prolonged increase in the plasma amino nitrogen curve of uremic patients fed 25 Gm. of glycine than in that of normal persons. However, in nephritic patients in whom the nephrotic syndrome was the outstanding feature of the disease, no abnormality in the plasma amino nitrogen curve was found after the feeding of glycine. Ludewig, Williams, and Chanutin[14] found a direct relation between protein intake and blood urea elevation in rats with varying degrees of reduction in renal function. Farr and Smadel[15] found that rats with nephritis produced by injecting anti-rat-kidney serum tended to recover when maintained on a high carbohydrate-low protein diet, while a medium protein diet frequently was associated with chronic nephritis and death from renal insufficiency. Such rats fed a high protein-low carbohydrate diet invariably developed a chronic progressive nephritis and the mapority of these animals died of renal failure.

In 1939 Addis and Lew[16] reported that acute renal insufficiency produced by ligation of the vena cava above the kidneys was more frequently fatal for animals previously maintained on a high meat diet than when the diet had been low or moderate in protein content. Later it was shown[17] that rats with one fourth the normal amount of renal tissue underwent glomerular and tubular damage when maintained on a diet moderate-to-rich in protein. If a high protein diet was given immediately after subtotal nephrectomy the group died, while those given a diet low in protein survived. More rigidly controlled studies of a similar nature were reported by Addis and associates[18] who fed isocaloric diets of varying protein content to rats following subtotal nephrectomy. The mortality rate increased with the protein consumption from nil for rats eating essentially no protein to 43 per cent within a week following operation for those consuming 2.3 Gm. of protein daily. Swift[19] studied the effect of protein intake upon the course of nephritis produced in three strains of rats by injection of nephrotoxic serum. A high protein diet was found to be conducive to chronic nephritis in all three strains though variations in susceptibility were noted.

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Lippman and Persike[20] reported a reduction in azotemia and creatininemia in twenty-one uremic patients maintained on a low protein diet (0.5 Gm. per kilogram per day plus an amount equal to the protein lost in urine). Borst[21] feels that prevention of infection and maintenance of patients on a low protein-high carbohydrate diet are measures which tend to postpone the development of uremia in patients with advanced renal impairment.

In 1939, Bergman and Drury[22] wrote that the low variability of the survival time of the standard nephrectomized rat makes it an excellent test object for the study of the toxic mechanisms in renal insufficiency and for the study of the efficacy of different therapeutic measures in this condition. They stated that meat is definitely toxic to the nephrectomized rat, whether fed before or after the kidneys are removed. Mylon and Goldstein,[23] in 1948, reported the production of uremia in two groups of dogs by renal artery ligation as well as by bilateral nephrectomy. One group was maintained prior to surgery on a carbohydrate diet, the other on a protein diet. Following either operative procedure the nitrogen retention was more rapid, the toxic symptoms were more severe, and the survival time was shorter in protein prefed animals as compared with the carbohydrate group. In another report Mylon, Smith, and Goldstein[24] showed that marked signs of toxicity followed ingestion of the high protein diet in nephrectomized dogs and in those with reduced renal function. Intrajejunal administration of Amigen or glycine in amounts corresponding to a high protein diet resulted in a significant reduction in the survival time of nephrectomized dogs. Masson, Corcoran, and Page[25,26] in 1948 reported that the administration of protein diets to rats following bilateral nephrectomy lowered their survival time.

The foregoing résumé of the literature indicates that the clinical and laboratory findings characteristic of uremia are intensified by protein administration. However, there is inadequate experimental evidence as to whether or not parenteral protein hydrolysates or amino acids definitely influence the survival time of anuric animals. This question is of practical importance in the management of patients with acute or chronic renal insufficiency. The following experiments were undertaken to provide further information on this question. The purpose of this study was to measure the effect of several protein hydrolysate solutions, as well as specific amino acids, on the survival time and blood chemistry of the anuric rat.

METHODS

The rat was chosen as the experimental animal because of its availability and uniformity of response. There were two major groups of rats. Group A rats consisted of Sprague-Dawley 250 to 300 gram. female rats. Group B rats consisted of Sprague-Dawley 350 to 400 gram discarded female breeder rats. With the exception of one group, all of the rats were rendered anuric by bilateral ureteral ligation. The rats were maintained preoperatively for a week or longer on the standard diet of Purina Chow biscuits, horse meat, bread, and water. Surgery was done under ether anesthesia without using any aseptic technique. The ureters were mobilized, doubly ligated, and severed through a one-inch midline incision in the anterior wall of the abdomen. After surgery the rats received no food. They were allowed water ad libitum. Survival times were measured in hours from the time of surgery to the time of death. This was accurate on most rats to within two hours.

All the substances to be tested were administered by intraperitoneal injection without aseptic technique. This means was considered to be accurate and effective after testing two groups of five normal rats each by giving them daily intraperitoneal injections of 10 per cent dextrose and of 10 per cent amino acid solution (Merck) respectively for a period of three weeks in volumes gradually increasing to 20 c.c. per injection per day. None of the rats suffered any apparent ill effects. Post-mortem examination revealed relatively little reaction of the peritoneum and omentum with only a moderate amount of adhesion formation.

Blood specimens were obtained for analysis by drawing cardiac blood from the rat when lightly anesthetized under ether. An oiled syringe and a 20-gauge needle were used. By this method 10 to 17 c.c. of blood usually could be obtained. Blood specimens from every two rats were pooled in order to given adequate amounts of serum for all the determinations. Most of the blood samples were analyzed for serum calcium, serum inorganic phosphorus, sodium, potassium, amino and nonprotein nitrogen.

Serum calcium was determined according to the method of Kramer and Tisdall[27] as modified by Clark and Collip[28] by using the wash solution of Wang,[29] and further modified according to the practice of the laboratory of using round-bottomed centrifuge tubes and four hours of precipitation.

Serum inorganic phosphorus was analyzed by the method of Kuttner and Lichtenstein[30] with the modification of Gomori.[31]

Serum sodium and potassium determinations were done on the Beckman flame photometer.

Amino nitrogen was determined on the Folin-Wu filtrate by the method of Frame, Russell, and Wilhelmi[32] as modified by Russell.[33]

Nonprotein nitrogen was analyzed on the Folin-Wu filtrate according to the method of Koch and McMeekin.[34]

Materials Used and Dose.—Four different protein hydrolysates and thirteen individual amino acids were studied. The protein hydrolysates are as follows: (1) 10 per cent amino acid solution (Merck)* which consists of a mixture of free amino acids, glutamic acid and aspartic acid having been removed; (2) 10 per cent casein-enzyme hydrolysate (Mead Johnson 10 per cent Amigen)† which is an enzymatic (pancreatic) hydrolysate of casein; (3) 10 per

TABLE I. COMPOSITION OF PROTEIN HYDROLYSATE SOLUTIONS

	10% AMINO ACID SOL.*	10% CASEIN ENZYME HYDROL.†	10% CASEIN ACID HYDROL.‡	10% PLASMA HYDROL.§
pH	5.93	5.63	4.13	5.68
Na content (meq./L.)	14.5	97.0	4.7	220.
K content (meq./L.)	0.26	1.26	0.51	12.5
Nitrogen (Gm./L.)	14.4	12.0	14.0	12.0
Essential amino acids (mg./c.c.)				
Arginine	1.90	3.6	3.9	4.0
Histidine	4.30	1.9	1.6	2.2
Isoleucine	7.80	6.0	6.1	3.2
Leucine	16.70	9.0	11.6	8.0
Lysine	10.30	5.7	8.2	7.4
Phenylalanine	3.70	4.9	3.6	3.6
Methionine	4.10	2.6	3.6	1.2
Threonine	1.80	2.9	2.9	5.6
Tryptophane	1.01	1.2	1.0	1.0
Valine	6.70	5.4	7.3	6.2
Other amino acids (mg./c.c.)				
Glycine	23.0	0.4		
Glutamic acid		17.1		11.6
Proline		6.2		
Serine		4.9		
Aspartic acid		4.7		8.6
Alanine		4.2		
Hydroxyproline		1.5		
Cystine		0.3		2.2
Tyrosine				1.0

*Merck's 10 per cent amino acid solution.
†Mead Johnson's 10 per cent Amigen.
‡Winthrop-Stearn's 15 per cent Parenamine (diluted to 10 per cent).
§Baxter's 10 per cent protein hydrolysate.

*Supplied by Merck & Company, Inc.
†Supplied by Mead Johnson & Company.

cent casein-acid hydrolysate (Winthrop-Stearns 15 per cent Parenamine diluted to 10 per cent) which is an acid hydrolysate of casein from which the excess salts (sulfates, phosphates, etc.) have been removed and which is fortified with tryptophane[35]; and (4) 10 per cent plasma hydrolysate [Baxter's 10 per cent protein hydrolysate (C9-99)]* which is an enzymatic digest of bovine plasma. The composition of these hydrolysates according to the manufacturers is shown in Table I.

The specific amino acids† used were L(−) leucine, glycine, L(+) arginine, L(+) lysine, L(+) glutamic acid, L(+) histidine, L-phenylalanine, DL-phenylalanine, DL-valine, DL-isoleucine, DL-threonine, DL-alanine, DL-methionine, and DL-tryptophane. Of these the first seven were the naturally occurring forms and the last seven were the racemic forms.[36]

Dextrose was used in 10 per cent solution and given to anuric rats as control for the volume of fluid injected and to simulate the osmotic characteristics of the tested solution. Five and 10 per cent urea in 5 per cent dextrose solutions were used as a further control measure and injected in dosages so as to give an amount of nitrogen equal to that present in the protein hydrolysate solutions.

The usual dose of protein hydrolysate solutions was 30 c.c. of a 10 per cent solution per kilogram body weight per day (or 3 Gm. per kilogram per day). Some rats were also given twice this dosage (6 Gm. per kilogram per day). The amino acids were given in equal molar dosages. This was arbitrarily set at 13.8 c.c. of 1.33 molar solution per kilogram per day. Because of their low solubility, it was impossible to give this dose of five of the amino acids without making the volume of injection too great. These five were DL-isoleucine, L(−) leucine, DL-phenylalanine, L-phenylalanine, and DL-tryptophane. Consequently, the above-mentioned amino acids were given in half the dose (6.9 c.c. of 1.33 molar solution per kilogram per day). Most of the injections were less than 20 c.c. per day.

RESULTS

A large number of control animals receiving no injections after bilateral ureteral ligation were run in each major group of rats (Groups A and B). Approximately five rats out of each shipment of fifty coming to the laboratory

TABLE II. SURVIVAL TIMES OF UREMIC RATS GIVEN DAILY INJECTONS OF PROTEIN HYDROLYSATES

	GROUP B RATS				GROUP A RATS			
	NUMBER OF RATS	MEAN SURVIVAL (HR.)	STD. DEV. (HR.)	T-RATIO FROM CONTROL	NUMBER OF RATS	MEAN SURVIVAL (HR.)	STD. DEV. (HR.)	T-RATIO FROM CONTROL
DOSE EQUALS 3 GM./KG./DAY								
Control rats	67	87.95	12.99		61	75.70	13.1	
10% Dextrose	10	86.80	7.46	0.35	18	69.55	9.69	1.85
10% Plasma hydrol.	30	79.61	16.61	2.67				
10% Casein-enzyme hydrol.	30	78.04	13.03	3.47				
10% Casein-acid hydrol.	30	61.65	11.77	9.48				
10% Amino acid solution	10	58.60	15.38	6.96	21	51.25	15.38	7.05
DOSE EQUALS 6 GM./KG./DAY								
Control rats	67	87.95	12.99		61	75.70	13.1	
10% Dextrose					20	65.34	11.31	3.17
10% Urea in 5% dextrose	10	62.10	4.11	6.21	10	56.26	9.58	4.49
10% Casein-acid hydrol.	15	22.96	8.35	18.49				
10% Amino acid solution					20	33.63	11.48	12.83

*Supplied by Baxter Laboratories, Inc.

†All except 1-phenylalanine were supplied by Merck & Company, Inc.

were taken as controls. In Group A (250 to 300 gram rats) there were sixty-one controls and the average survival time was 75.70 hours with a standard deviation of 13.1 hours. In Group B (350 to 400 gram rats) there were sixty-seven controls and the average survival time was 87.95 hours with a standard deviation of 12.99 hours. These two groups varied significantly from each other (t-ratio = approximately 4.0). However, within each specific group there was no correlation between the weight of the animal and the survival time (in Group A, r = 0.047; in Group B, r = 0.062). The survival times of all rats given intraperitoneal injections were compared with the control values for that group.

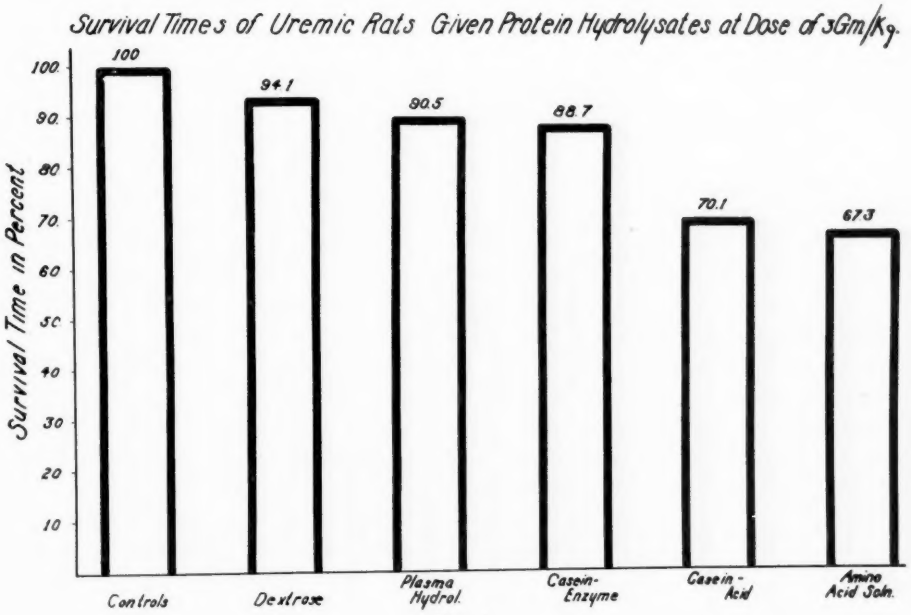

Fig. 1.

The first series of anuric rats was given daily intraperitoneal injections of 3 Gm. per kilogram per day of the various protein hydrolysates. Dextrose was given in the same dosage to serve as a further control. The results are listed in Table II and Fig. 1. The survival time for the rats receiving 10 per cent dextrose was 5.9 per cent less than the controls; this difference is not significant. Rats receiving 10 per cent plasma hydrolysate or 10 per cent casein-enzyme hydrolysate had survival times of 9.5 per cent and 11.3 per cent respectively, less than that of the controls. This difference is quite significant and this is also significantly less than the group of rats receiving 10 per cent dextrose. However, the plasma-enzyme hydrolysate group and the casein-enzyme hydrolysate group did not vary significantly from each other. The average survival times of rats receiving 10 per cent casein-acid hydrolysate or 10 per cent amino acid solution was less than the controls by 29.9 per cent and 32.7 per cent respectively. These two values are significantly less than the controls as well as significantly less than the enzyme hydrolysate groups.

The difference in effect on survival time of uremic rats between casein-acid hydrolysate and amino acid solution on the one hand and the plasma-enzyme and casein-enzyme hydrolysate on the other was quite constant. Ten rats in each group were operated on several months after the first twenty rats in each group had been completed and the same relative effect was observed.

In the second series, the anuric rats were given daily intraperitoneal injections of two protein hydrolysates and dextrose in twice the dosage used in the first series (i.e. 6 Gm. per kilogram per day). Also, 10 per cent urea in 5 per cent dextrose was given to a group of rats so that they received an amount of nitrogen equal to that given to the rats injected with protein hydrolysates. The results of these data are recorded in Table II and in Fig. 2. The survival time of rats receiving 6 Gm. per kilogram of dextrose daily was slightly but not significantly

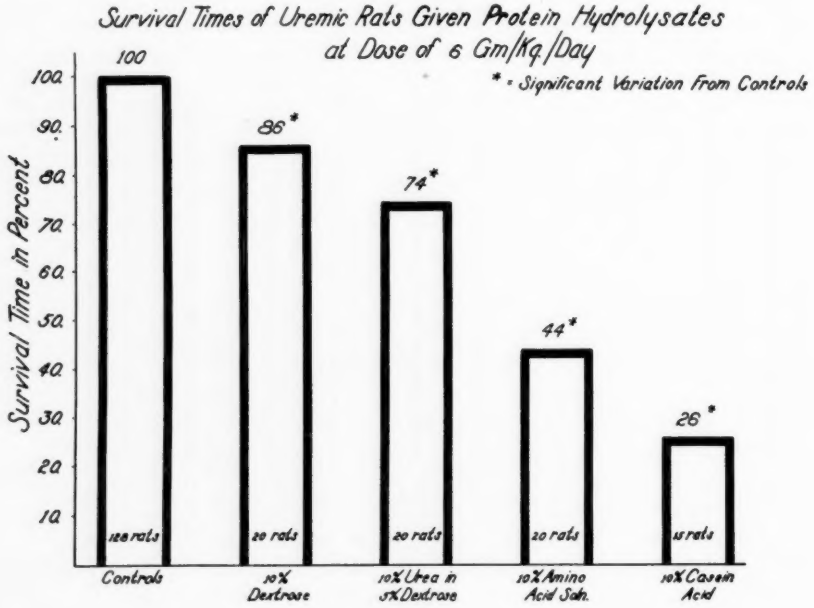

Fig. 2.

less than the previous series receiving 3 Gm. per kilogram, but was significantly less than the controls. Rats receiving 10 per cent amino acid solution or 10 per cent casein-acid hydrolysate survived 66 per cent and 37 per cent as long, respectively, on a dose of 6 Gm. per kilogram per day as they did on 3 Gm. per kilogram per day. On the other hand, the rats receiving 10 per cent urea in 5 per cent dextrose survived about twice as long as those receiving amino acid solution or casein-acid hydrolysate, and only 26 per cent less time than the controls.

The data indicate that a greater effect on the survival time of the uremic rats was caused by the amino acid solution and casein-acid hydrolysate than by plasma-enzyme hydrolysate or the casein-enzyme hydrolysate. Also the survival

time decreases as the dose of administration of protein hydrolysate increases. That this is not due to increased urea formation is demonstrated by the longer survival time of rats that received injections of urea.*

A third series of rats was run in which blood specimens were drawn for chemical analysis approximately forty-eight hours after bilateral ureteral ligation. These rats were given protein hydrolysates or dextrose and urea at a dose of 3 Gm. per kilogram. Two injections were given, one immediately after surgery and the second about twenty-four hours after surgery. Determinations on control rats (bilateral ureteral ligation but no injections) and normal fasting rats were also made. All the rats at the time of obtaining the specimens were fairly strong and active. However, the rats receiving the amino acid solution were weaker and less active than the remaining groups. The results are listed in Table III. Even though the series is too small for accurate statistical analysis, certain trends can be seen. The most striking observation is the slower elevation of inorganic phosphorus and nonprotein nitrogen (13.6 mg. per cent and 188 mg. per cent respectively) of those that received 10 per cent dextrose injections as compared with the control rats that received no injections (18.2 mg. per cent and 258 mg. per cent respectively). The plasma hydrolysate and amino acid solution groups showed the highest values for inorganic phosphorus, the lowest values for serum calcium, relatively no change in sodium concentration, only a slight elevation in potassium concentration, and the highest elevations for both amino nitrogen and nonprotein nitrogen. In spite of the fact that a significant difference in survival time occurs between anuric rats given plasma hydrolysate and those given amino acid solution, no differences of any significance are demonstrated by the chemical data. The rats that received glucose or urea plus glucose did not show as much hypocalcemia and hyperphosphatemia as those given protein hydrolysates.

A fourth small series was done in which blood specimens were drawn approximately twenty-four hours after bilateral ureteral ligation. These rats were given injections of dextrose, urea and dextrose, or amino acid solution in dosages of 6 Gm. per kilogram. Two injections were given, the first one immediately after surgery and the second approximately twenty hours postoperatively. In this series, the rats that were given amino acid solution were very weak and ill in twenty-four hours; some were moribund. The remaining groups of rats were in considerably better condition symptomatically. Only serum potassium and nonprotein nitrogen determinations were made in this series. Less elevation of nonprotein nitrogen was again noted in rats that received 10 per cent dextrose (159 mg. per cent) than in the controls (174 mg. per cent). The nonprotein nitrogen levels in rats given urea and amino acid solution (375 mg. per cent and 340 mg. per cent respectively) were two or more times as great as that seen in the controls or the dextrose-treated rats. A significant elevation of serum potassium (7.6 meq. per liter) was observed in the rats given amino acid solution as compared with the remaining groups. However, this level is not as high as that listed by Hoff, Smith, and Winkler[37] as necessary to cause death in the normal or the uremic dog.

*No rats were done in which glucose was added to the protein hydrolysate.

TABLE III. CHEMISTRY ON CARDIAC BLOOD OF UREMIC RATS

	48 HR. AFTER URETERAL LIGATION TWO INJECTIONS AT DOSE OF 3 GM./KG./DAY												
	NUMBER OF RATS	INORG. P (MG. %)	RANGE OF VALUES	CA (MG. %)	RANGE OF VALUES	NA (MEQ./L)	RANGE OF VALUES	K (MEQ./L)	RANGE OF VALUES	AMINO N (MG. %)	RANGE OF VALUES	N.P.N. (MG. %)	RANGE OF VALUES
Normal rats*	6	5.8	5.3- 6.3	10.46	10.32-10.59	137.5	137.5	4.3	4.3	13.8	13.5-14.0	38.9	30.2-47.6
Control rats†	7	18.2	18.0-26.0	10.66	10.40-11.07	133.6	125-137.5	5.1	4.7-5.4	15.6	13.0-17.0	258	238-287
10% Dextrose†	10	13.6	11.3-16.5	10.74	10.59-10.88	133	130-137.5	4.9	4.4-5.3	15.8	14.2-16.5	188	143-224
5% Urea in 5% dextrose†	10	15.2	13.5-16.7	9.99	9.55-10.50	128	120-130	4.7	4.4-5.2	17.0	15.6-18.2	312	310-320
10% Plasma hydrol.†	10	20.9	18.0-26.3	8.86	7.85-9.46	138	136-144	4.9	4.4-5.3	17.7	16.1-19.9	357	306-418
10% Amino acid solution†	10	19.2	16.0-21.7	8.52	8.13-8.75	133	130-135	5.0	4.5-5.4	19.0	18.2-19.7	381	341-420

	24 HR. AFTER URETERAL LIGATION TWO INJECTIONS AT DOSE OF 6 GM./KG./DAY				
	NUMBER OF RATS	K (MEQ./L)	STD. DEV.	N.P.N. (MG. %)	STD. DEV.
Control rats‡	10	5.9	0.62	174	25.69
10% Dextrose‡	10	5.7	0.78	159	15.54
5% Urea in 5% dextrose‡	10	5.7	0.81	375	25.31
10% Amino acid sol.‡	18	7.6	1.29	340	36.84

*Blood specimens pooled from every three rats.
†Blood specimens pooled from every two rats.
‡Blood specimens not pooled.

Specific Amino Acids.—A fifth and sixth series of rats were included in which intraperitoneal injections of specific amino acids were given after bilateral ureteral ligation. The ten essential amino acids as listed by Rose[38] were studied as well as three nonessential amino acids (glycine, DL-alanine, and L(+) glutamic acid). They were given in equimolar dosages. The dose in the first series was equivalent to 13.8 c.c. of 1.33 molar solution per kilogram per day. The concentration of each specific amino acid varied in relation to its solubility in distilled water. All the solutions were prepared just prior to injection. The results are listed in Table IV and Fig. 3. Twenty rats given 7 per cent DL-valine after bilateral ureteral ligation survived for an average of 87.19 hours which is essentially the same as the controls. Rats given a solution of 3 per cent DL-isoleucine plus 3 per cent L(−) leucine survived 90 per cent as long as the controls (Fig. 3), this difference not being significant. The survival time of groups given 8 per cent DL-threonine, 11.9 per cent DL-alanine, and 10 per cent glycine was 83 per cent, 75 per cent, and 65 per cent respectively of that of the controls. All of these differences are statistically significant. The threonine and glycine groups did not vary significantly from the alanine group but did differ significantly from each other. A marked decrease in survival time was observed by groups of rats that received 14 per cent L(+) arginine, 13.4 per cent L(+) lysine, 8 per cent DL-methionine, 5 per cent L(+) glutamic acid, and 14 per cent L(+) histidine. These groups, with the exception of histidine, survived 20 per

TABLE IV. SURVIVAL TIMES OF UREMIC RATS GIVEN DAILY INJECTIONS OF SPECIFIC AMINO ACIDS

	DOSE EQUIVALENT TO 6.9 C.C. 1.33 MOLAR SOL./KG./DAY							
	GROUP B RATS							
	NUMBER OF RATS	MEAN SURVIV. (HR.)	STD. DEV.	T-RATIO FROM CONTROL				
Control rats	67	87.95	12.99					
DL-valine 7%	20	87.19	8.30	0.12				
L(−) leucine 3% plus DL-isoleucine 3%	10	78.97	6.71	1.93				
DL-threonine 8%	20	72.90	10.88	4.32				
DL-alanine 11.9%	20	65.20	16.67	5.92				
Glycine 10%	16	57.14	3.13	8.75				
L(+) arginine 14%	10	17.89	5.36	15.74				
L(+) lysine 13.4%	9	17.19	3.61	15.19				
DL-methionine 8%	20	14.31	5.04	23.65				
L(+) glutamic acid 5%	20	12.86	5.12	24.81				
L(+) histidine 14%	10	3						
	DOSE EQUIVALENT TO 6.9 C.C. 1.33 MOLAR SOL./KG./DAY							
	GROUP B RATS				GROUP A RATS			
	NUMBER OF RATS	MEAN SURVIV. (HR.)	STD. DEV.	T-RATIO FROM CONTROL	NUMBER OF RATS	MEAN SURVIV. (HR.)	DEV. STD.	T-RATIO FROM CONTROL
Control rats	67	87.95	12.99		61	75.70	13.1	
DL-isoleucine 3.5%	9	82.35	12.74	1.07				
L(−) leucine 3.5%	5	69.18	15.97	2.43	20	66.58	12.13	2.75
L-phenylalanine 3.5%	20	45.87	6.31	13.98				
DL-phenylalanine 3.5%	20	39.94	10.84	14.02				
L(+) arginine 14%	10	32.50	3.16	12.54				
DL-tryptophane 4%	20	26.11	12.49	17.60				

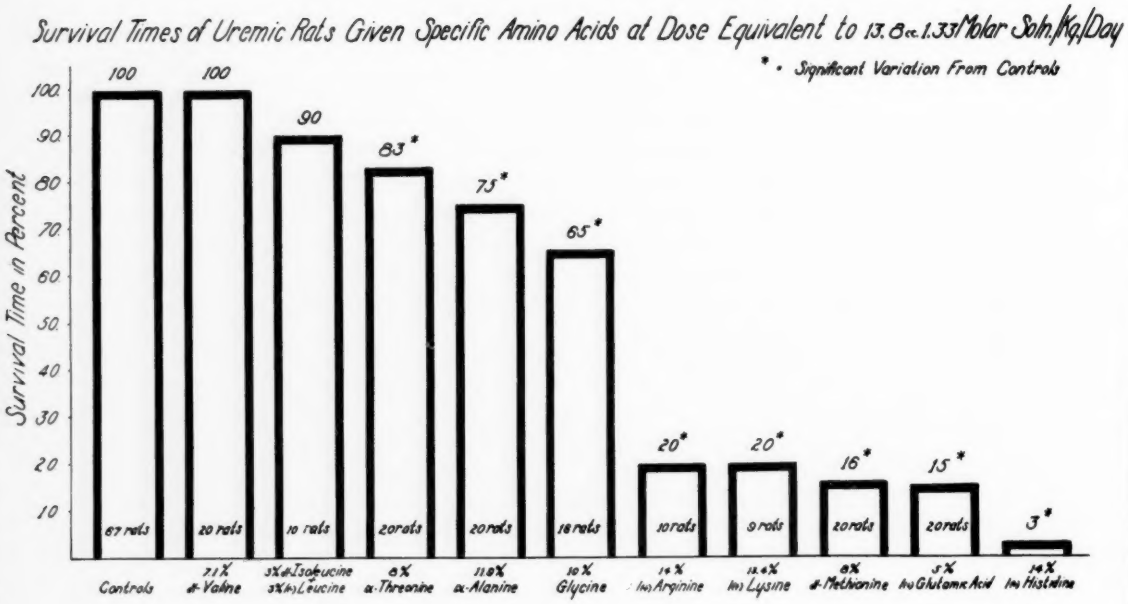
Survival Times of Uremic Rats Given Specific Amino Acids at Dose Equivalent to 13.8 cc. 1.33 Molar Soln./Kg./Day
* = Significant Variation From Controls
Survival Time in Percent
100
90
80
70
60
50
40
30
20
10
100
100
90
83*
75*
65*
20*
20*
16*
15*
3*
67 rats
20 rats
10 rats
20 rats
20 rats
18 rats
10 rats
9 rats
20 rats
20 rats
3 rats
Controls
7.1% dl-Valine
3% dl-Isoleucine 3% (l+) Leucine
8% α-Threonine
11.8% α-Alanine
10% Glycine
1% (l+) Arginine
13.4% (l+) Lysine
8% dl-Methionine
5% (l+) Glutamic Acid
1% (l+) Histidine

Fig. 3.

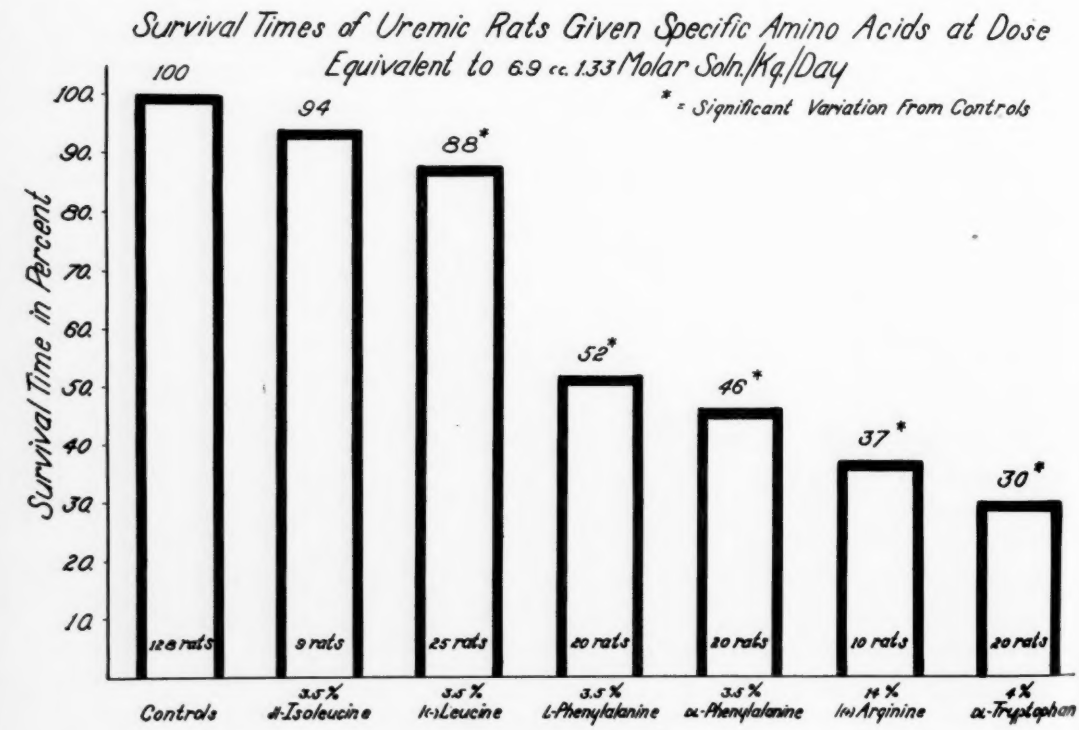
Survival Times of Uremic Rats Given Specific Amino Acids at Dose Equivalent to 6.9 cc. 1.33 Molar Soln./Kg./Day
* = Significant Variation From Controls
Survival Time in Percent
100
90
80
70
60
50
40
30
20
10
100
94
88*
52*
46*
37*
30*
128 rats
9 rats
25 rats
20 rats
20 rats
10 rats
20 rats
Controls
3.5% dl-Isoleucine
3.5% (l+) Leucine
3.5% L-Phenylalanine
3.5% α-Phenylalanine
1% (l+) Arginine
4% α-Tryptophan

Fig. 4.

cent, 20 per cent, 16 per cent, and 15 per cent respectively of the time of their controls, but did not vary significantly from each other. The histidine group survived for less than three hours which is significantly less than for all the other amino acid groups.

Because of their low solubilities, five amino acids were given to anuric rats at a dose equivalent to 6.9 c.c. of 1.33 molar solution per kilogram per day, and the results are listed in Table IV and Fig. 4. It was necessary to inject most of these amino acids in a warm supersaturated state. Rats given 3.5 per cent DL-isoleucine survived 94 per cent as long as the controls (Fig. 4), a difference which is not significant. A separate group of twenty uremic rats not listed here, when given injections of isoleucine in just half this dose, survived for an average of 91.4 hours. The group given leucine survived 88 per cent as long as the controls, a difference which is statistically significant. Decreased survival times were manifested by the groups that were given 3.5 per cent L-phenylalanine and 3.5 per cent DL-phenylalanine (52 per cent and 46 per cent as long as the controls, respectively). The difference between these two groups is just barely significant. Shorter survival times were manifested by the groups injected with 14 per cent L(+) arginine and 4 per cent DL-tryptophane (37 per cent and 30 per cent as long as the controls, respectively). These values were significantly less than for the other amino acids given at this concentration but not significantly different from each other.

DISCUSSION

The amount of glucose administered (3 to 6 Gm. per kilogram per day) did not prolong the survival time of the uremic rat, although it did give evidence of retarding the elevation of the serum inorganic phosphorus and nonprotein nitrogen as compared with the controls. Other investigators have reported[23, 25, 26, 42] that a high carbohydrate diet will prolong the survival time of nephrectomized animals. The control rats received no calories orally or parenterally in the postoperative period and, therefore, had to derive their entire daily carbohydrate needs from the breakdown of glycogen, fat, and protein, which resulted in a more rapid elevation of the inorganic phosphorus and the nonprotein nitrogen. There is relatively little increase in the amino nitrogen of the blood stream in comparison to the amounts administered. This fact indicates that the tissues of the body still retained their capacity to store or catabolize large amounts of these substances. The fact that injection of amino acid solution resulted in the greatest average rise in blood amino acid nitrogen may be due to its greater free amino acid content or to the proportions in which the amino acids are present in the solution. The data are too few to enable one to determine whether or not the shorter survival time associated with injection of the amino acid solution can be correlated with the greater average elevation in blood amino nitrogen values obtained for this group. The slower increase in inorganic phosphorus and nonprotein nitrogen in glucose-injected animals is evidence that protein catabolism was retarded. This fact, taken with the lack of effect of glucose on survival time, suggests that protein catabolism was not the limiting factor in determining the survival time of ureteral ligated rats.

Injections of plasma-enzyme hydrolysate and casein-enzyme hydrolysate had a significant but much less marked effect on survival time than casein-acid hydrolysate or the amino acid solution, which shortened the survival time to approximately two-thirds that of the control groups. The reason for this difference is not clear and cannot be related to the blood chemical values. The results suggest that not only the amount but also the chemical state and proportions of the amino acids influence their effect on survival time and presumably their fate in the body. It was considered that the toxicity of the protein hydrolysates may have been due to certain of the specific amino acids which they contain. It was demonstrated that some amino acids, when given in equimolar dosages, have a greater effect on survival of the anuric rat than have other amino acids. As further evidence as to whether or not individual amino acids contributed to the toxicity of a given mixture, ureteral ligated rats were given intraperitoneal injections of amino acids in dosages proportionate to their concentration in the amino acid solution (Merck). The amino acids which were present in the highest concentration (glycine 23 per cent, leucine 16 per cent, lysine 10 per cent) significantly lowered the survival times, whereas those of lower concentration (isoleucine 8 per cent, histidine 4 per cent, arginine 2 per cent) had no significant effect on the survival time. These findings indicate that the reduced survival time produced by this mixture is due to several amino acids present in high concentration rather than to high toxicity of a single amino acid.

The amino acids tested when given in equimolar dosages may be divided roughly into three groups: (A) Those causing little or no effect on the survival time, (B) those causing a moderate effect, and (C) those causing a marked effect. In such a classification they are listed as follows:

A. Little or no effect.
   1. DL-valine
   2. DL-isoleucine
   3. L(−)leucine

B. Moderate effect.
   1. DL-threonine
   2. DL-alanine
   3. Glycine
   4. L-phenylalanine
   5. DL-phenylalanine

C. Marked effect.
   1. L(+) arginine
   2. DL-tryptophane
   3. L(+) lysine
   4. DL-methionine
   5. L(+) glutamic acid
   6. L(+) histidine

The question arose regarding the tolerance of normal rats to injections of some of the amino acids. Four amino acids, glycine, leucine, histidine, and

glutamic acid, were tested on normal rats. Rats tolerated injections of 10 per cent glycine quite well when they were less than 25 c.c. of 1.33 molar solution per kilogram per day. However, as the dose rose to 30 c.c. of 1.33 molar solution per kilogram per day or more, the rats would die. The actual dosage of glycine given to the uremic rats was 13.8 c.c. of 1.33 molar solution per kilogram per day. Rats that received 2.4 per cent leucine easily tolerated dosages up to 50 per cent larger than those given to the uremic rats. Histidine (10 per cent) became toxic to the normal rat when the injections became 50 per cent greater than those given to the uremic rats. Five per cent glutamic acid was toxic to rats when given in dosages equal to those given to the uremic rats. Consequently, in dosages approximating those given to the uremic rats, only glutamic acid and histidine had a toxic effect on the normal rats.

The pH of the various solutions ranged from 3.96 for glutamic acid to 6.38 for leucine. With the possible exception of glutamic acid and histidine, there was no relationship between the pH of the solutions and the survival times. There was no correlation between the amount of nitrogen injected and the survival time. Also, no correlation existed between the molecular weights of the amino acids and survival time.

Because of the great expense of the natural forms of some amino acids, it was necessary in some instances to use the racemic forms. However, the responses of uremic rats to injections of DL-phenylalanine and L-phenylalanine were compared. Twenty rats that were given L-phenylalanine survived for an average of 45.87 hours, while those injected with DL-phenylalanine survived for an average of 39.94 hours (Table IV). The difference between the two groups is barely significant ($t = 2.1$). Therefore, in this instance the D-form appears to be only slightly more toxic than the L-form. Other workers[39, 40, 41] have presented some evidence suggesting that the unnatural isomers of amino acids are more toxic than the natural forms. However, absolute evidence of this difference has not been demonstrated.

Another question to be answered was a possible difference of response to protein hydrolysates of rats that have had bilateral ureteral ligation as compared to those with bilateral nephrectomy. Twenty rats were made anuric by nephrectomy and given daily injections of casein-enzyme hydrolysate at a dose of 3 Gm. per kilogram per day. The average survival time was 81.52 hours. The average survival time of rats made anuric by ureteral ligation and receiving the same injections was 78.04 hours. The difference between these two values is not significant. Nephrectomy probably does not alter the response to protein hydrolysates as compared with ureteral ligation.

SUMMARY

Protein hydrolysates and a mixture of amino acids all reduced the survival time of anuric rats when given at a dose of 3 Gm. per kilogram per day.

Casein-acid hydrolysate and amino acid solution caused a greater decrease in the survival time of the uremic rat than plasma-protein enzyme hydrolysate or casein-enzyme hydrolysate, when given in doses of 3 Gm. per kilogram per day.

As the amount of protein hydrolysates administered to anuric rats is increased, the survival time decreases. This is not due to urea formed from the protein hydrolysates.

A slower elevation of inorganic phosphorus and nonprotein nitrogen resulted after injections of glucose (3 to 6 Gm. per kilogram per day) as compared with control rats that received no injections. However, there was no difference in the survival time of these animals.

Thirteen different amino acids injected into uremic rats produced from slight to marked effects upon survival time.

The survival time of rats made anuric by ureteral ligation or nephrectomy is influenced to a similar degree by injections of protein hydrolysate.

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A FAT EMULSION FOR INTRAVENOUS NUTRITION IN RABBITS

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RECENTLY our work has led to the development of a stable, fine fat emulsion which can be given intravenously for nutritional purposes.[1] This preparation, which has been administered intravenously to dogs for as long as ten weeks, did not produce any toxic effects and the experimental evidence demonstrated the utilization of the infused fat for energy in the body.[1, 2, 3] Results obtained by other investigators concerning the utilization of the intravenously administered fat have also supported similar conclusions.[4-7] However, further information on the toxic effects which might follow intravenous administration of this fat emulsion on animals other than dogs is quite desirable before it can be used with confidence clinically.

The experiments carried out in this report were undertaken in an effort to study the effect of the fat emulsion used in this laboratory on arterial blood pressure, antigenic and pyrogenic reactions in rabbits. The effects of the daily infusion of 2.0 Gm. of fat per kilogram of body weight on hematologic, histologic, and body weight changes were also carefully investigated.

EXPERIMENTAL PROCEDURES AND RESULTS

Rabbits weighing 1.4 to 3.0 kilograms were used. There was no selection of strains and both males and females were included in the experiments. The animals were fed Purina rabbit chow and greens. Carrots were given also twice a week. They were kept under observation for seven to fourteen days prior to use for experiment. The preparation and composition of the fat emulsion were similar to that previously used.[3] Injections were made into the ear veins exclusively. A 10 per cent fat emulsion was used in all the experiments.

Effect of Intravenous Injection of Fat Emulsion on Blood Pressure.—In four unanesthetized rabbits and four rabbits under ether anesthesia, the intravenous injection of the emulsion (1.5 to 3.0 Gm. of fat per kilogram of body weight) produced a slight rise of 4 to 16 mm. Hg in the arterial blood pressure. The rise occurred during the injection and subsided immediately thereafter. The injection was completed in three or four minutes and the blood pressure was taken by inserting a 21 gauge hypodermic needle into the femoral artery which was connected with a mercury manometer through a three-way stopcock in all instances. In no instance was the change in arterial pressure significant.

*Antigenic Studies.**—Nine rabbits have been given 1.5 to 2.0 Gm. of fat per kilogram of body weight intravenously every day for three to four weeks. Blood sera were obtained from the rabbits on the tenth or eleventh day after

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*It is a pleasure to acknowledge the assistance and suggestions of Dr. Roy C. Avery in performing the antigenic tests.

the last injection. A precipitin test was carried out using twofold dilutions of the sera from 1:5 to 1:1,280. As a control, a test was also performed using blood sera which were obtained from three normal rabbits. Fat emulsion used for the test was diluted 1:100. The precipitin test was found to be negative in all cases. The animals showed no sign of hypersensitiveness following a large dose (40 c.c. of 10 per cent emulsion) of fat injected intravenously five weeks after the last injection. The fat emulsion is nitrogen free as shown by Kjeldahl determination.

Immediate Effects on Rectal Temperature and White Blood Cell Count (Pyrogen Test).—Forty rabbits were divided into eight groups of three to seven each. The animals were given intravenously 10 to 17 c.c. of fat emulsion per kilogram of body weight in two to five minutes. Rectal temperature and white blood cell count were determined before and one, two, and three hours after the injection of fat emulsion. The average increase of rectal temperature and change in white blood cell count are shown in Table I. It will be seen that the effect on rectal temperature seemed, at least in part, to depend on the age of the emulsion. Emulsions which were less than 2 months old were pyrogen free or very slightly positive, but those which were more than 2 months old produced a febrile reaction of more than 1° C. However, an emulsion could be rendered less likely to produce fever when reautoclaved. There was no significant change in white blood cell count in these experiments except that an average increase of 4,202 per cubic millimeter was observed (50 per cent increase) in the group of rabbits in which 6-month-old emulsion was injected.

Hematologic Observations and Body Weight.—After normal controls were observed for two weeks with red and white blood cell counts and determination of hemoglobin and body weight, twenty rabbits then received daily intravenous injections as follows. Two received 5 per cent glucose, five received the emulsifying agents (same amount and concentration as in the fat emulsion) in 5 per cent glucose, and thirteen received 10 per cent fat emulsion in 5 per cent glucose. The daily amount of fat given was 2.0 Gm. per kilogram body weight. Injections were continued for as long as four weeks after which the animals were sacrificed for histologic study. From Fig. 1, it will be noted that there was no significant change observed in the average weekly red blood cell count in any of the three groups of rabbits. In fact, a slight elevation was demonstrated during the last two weeks of infusion. Fig. 2 shows the average weekly determination of hemoglobin of the same groups of animals. Again no change occurred as a result of glucose, emulsifying agents, or fat emulsion administration. The average weekly white blood cell count showed considerable variation during the experimental period. A very slight increase might have occurred during the period of infusion in all the three groups of animals. The results are shown in Fig. 3.

Although balance studies of food intake and output were not carried out in all three groups of animals receiving glucose, emulsifying agents, or fat emulsion, they showed no apparent change of appetite and appeared in good spirits. Food was allowed ad libitum during the period of the daily infusions as well as during the previous control period. From Fig. 4 it will be seen that all three groups of animals demonstrated a slight gain in body weight.

TABLE I. EFFECTS ON RECTAL TEMPERATURE AND WHITE BLOOD CELL COUNT FOLLOWING INTRAVENOUS FAT ADMINISTRATION

AGE OF EMULSION	10 DAYS		1 MO.	3 MO.		4 MO.		5 MO.		5 MO. (REAUTOC.)	6 MO.		6 MO. (REAUTOC.)
NUMBER OF RABBITS	5		7	5		5		5		3	5		5
RECTAL TEMP. AND W.B.C. COUNT	RECTAL TEMP. °C	W.B.C. PER CU.MM.	RECTAL TEMP. °C	RECTAL TEMP. °C	W.B.C. PER CU.MM.	RECTAL TEMP. °C	W.B.C. PER CU.MM.	RECTAL TEMP. °C	W.B.C. PER CU.MM.	RECTAL TEMP. °C	RECTAL TEMP. °C	W.B.C. PER CU.MM.	RECTAL TEMP. °C
Before injection (average)	39.60	8,590	39.50	39.43	8,040	39.35	8,530	39.82	6,710	39.30	39.39	8,250	39.55
After injection (average) 1 Hr.	40.00	8,160	39.70	40.06	9,650	40.08	8,730	40.86	7,920	39.50	40.70	10,560	40.03
2 Hr.	40.16	10,040	40.20	40.62	7,130	40.93	8,940	41.18	6,220	40.57	41.06	15,875	40.98
3 Hr.	40.28	10,010	40.60	40.48	8,540	40.28	9,310	40.98	5,860	40.20	40.76	10,920	40.78
Average of 3 determinations	40.15	9,403	40.17	40.39	8,440	40.43	8,993	41.01	6,670	40.09	40.84	12,450	40.60
Average dif. (before and after injection)	+0.55	+813	+0.67	+0.96	+400	+1.08	+463	+1.19	-40	+0.79	+1.45	+4,202	+1.05
Change in W.B.C. (%)		+9.4%			+4.9%		+5.4%		-0.6%			+50%	

*Histologic Study.**—Postmortem examination revealed no gross abnormalities at the end of the four-week infusion period in all three groups of animals. Microscopically, only one rabbit (R6) of the fat emulsion infused group showed a few casts and two (R6 and R18) demonstrated very few fine fat globules in the collecting tubules of the kidney. There were also a few fine fat droplets scattered in the liver sinusoids in R18. Otherwise all other hematoxylin and

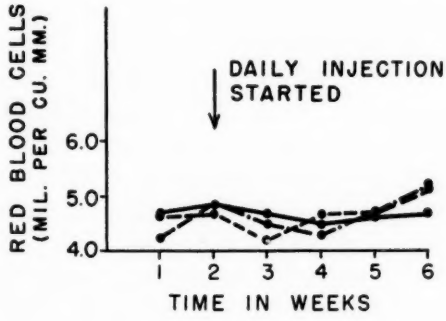

Fig. 1.

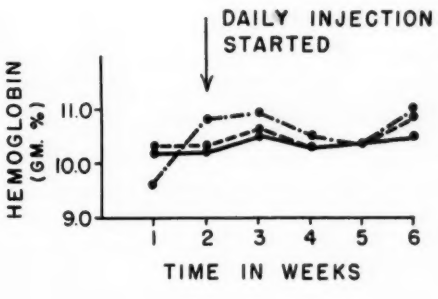

Fig. 2.

Fig. 1.—The average weekly red blood cell count. ●—•—●, Glucose-infused rabbits; ●-----●, emulsifying agents-infused rabbits; ●———●, fat emulsion-infused rabbits.

Fig. 2.—The average weekly blood hemoglobin determination. ●—•—●, Glucose-infused rabbits; ●-----●, emulsifying agents-infused rabbits; ●———●, fat emulsion-infused rabbits.

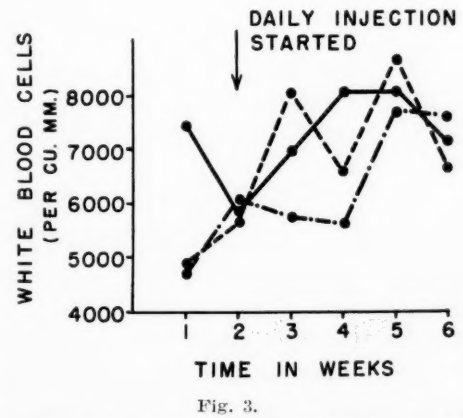

Fig. 3.

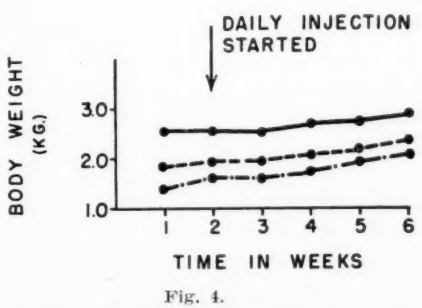

Fig. 4.

Fig. 3.—The average weekly white blood cell count. ●—•—●, Glucose-infused rabbits; ●-----●, emulsifying agents-infused rabbits; ●———●, fat emulsion-infused rabbits.

Fig. 4.—The change in body weight (average). ●—•—●, Glucose-infused rabbits; ●-----●, emulsifying agents-infused rabbits; ●———●, fat emulsion-infused rabbits.

eosin and sudan III stained sections of the liver, kidney, spleen, lung, and heart of the fat-infused animals showed no remarkable changes. In the animals receiving emulsifying agents, two (R1 and R12) showed hemosiderin in the spleen sections stained with hematoxylin and eosin and two (R3 and R12) demonstrated a few fine fat globules in the collecting tubule of the sudan III stained kidney

*I wish to acknowledge my indebtedness to Dr. John L. Shapiro of the Department of Pathology for interpretation of the histologic sections.

sections. Nothing otherwise significant was observed indicating pathologic changes. There was nothing obtained which appeared abnormal in the liver, kidney, lung, spleen, and heart sections of the glucose-infused animals.

DISCUSSION

The experimental observations presented in this paper indicate that fat emulsion prepared in this laboratory is nontoxic to rabbits. The slight elevation of arterial blood pressure during injection of fat emulsion is as one might expect. It was undoubtedly due to the intravenous administration of fluid resulting temporarily in an increase in the circulating blood volume which in turn caused the rise of blood pressure. A fall of blood pressure was never observed in spite of the presence of sodium cholate. The interpretation of this has been discussed previously.[1]

From the standpoint of clinical use, it is of extreme importance to know whether or not this fat emulsion is antigenic. It would not be suitable if it causes hypersensitivity. According to the nitrogen determinations using a modified Kjeldahl method, it can be stated with considerable confidence that the preparation is protein free. Because of the fact that Rosenau and Anderson[8] succeeded in one case in sensitizing with as minute an amount as 0.000001 c.c. of horse serum, further tests for antigen and sensitization appeared necessary. However, none of the nine rabbits showed a positive precipitin test even when the test was performed on the tenth or eleventh day after the last injection when precipitins are supposed to reach their highest concentration in the blood (eight to twelve days after the last injection). Furthermore, signs of anaphylactic shock or hypersensitiveness have never been observed in any of the tested rabbits. It is justifiable to conclude that the fat emulsions used in this laboratory were not antigenic.

Since the basic and extensive studies of Seibert[9, 10, 11] on pyrogens have been reported, it is a well-recognized fact that any medicinal agents or solutions intended for parenteral use and particularly for intravenous administration should be free of pyrogens or thermogenic substances. The most acceptable procedure is to take rectal temperatures at three successive hourly intervals after the injection of the test sample. In the work presented here, the white blood cell count was determined as well as the rectal temperature. The effect of pyrogen-containing solutions on the circulating white blood cell count was first demonstrated by Co Tui and associates.[12, 13] They reported the production of a marked leucopenia in addition to the elevation of body temperature in dogs and human subjects following intravenous injections of saline solutions which were made with boiled and filtered tap and aquarium waters. Chapman[14] also found that a marked leucopenia (a drop of 4,000 cells per cubic millimeter of blood or more) accompanied the fever reaction which occurred in injection of pyrogen-containing solutions. His studies were carried out on rabbits. The number of rabbits used to carry out the pyrogen test in this paper might be too small to reach a final conclusion. However, it is reasonable to state that emul-

sions which are less than 2 months old can be safely used for intravenous administration, and those which are more than 2 months old should be reautoclaved just before use.

It is interesting to point out that pyrogens cannot be destroyed by autoclaving at 12 pounds pressure for twelve minutes. Yet reautoclaving rendered the emulsions much less thermogenic. This leads me to believe that the febrile reaction following intravenous administration of an old fat emulsion must have been due at least in part to some other causes of unknown nature. Further investigation in this regard is undoubtedly necessary. As to the white blood cell count, leucopenia was never observed. In fact there was no significant variation in leucocyte counts demonstrated except that an average increase of 4,202 per cubic millimeter of blood was obtained following injection of a 6-month-old emulsion, which was approximately 50 per cent of the control finding. The average increase of rectal temperature for the same group of animals was 1.45° C. (Table I). The rise of white blood cell count was probably parallel with the increase of rectal temperature. It can be said that leucopenia probably cannot be used as a criterion for pyrogen test in our case.

The hematologic changes observed following intravenous administration of fat emulsion require no further discussion. Judging from the results presented in this paper, it can be confidently concluded that the daily infusion of 2.0 Gm. fat per kilogram of body weight as 10 per cent emulsion for as long as four weeks did not produce anemia. The change in white blood cell count might well be the daily variations. The gain in body weight further indicates the nontoxic nature of the preparation.

It is of interest to note that 2.0 Gm. of fat per kilogram of body weight in rabbits approximately corresponds to 130 Gm. of fat for an average-sized man. This amount of emulsion would furnish 1,400 calories per day.

It is difficult to determine whether or not the few fine fat globules observed in the collecting tubules of the kidneys of two of the thirteen emulsion-infused rabbits were the result of fat administration. However, since the same change was also observed in two of the five animals receiving emulsifying agents, it is reasonable to believe that the change was not due to fat infusion per se. Furthermore, it is of doubtful significance insofar as clinical use is concerned. This belief can be further supported by the fact that there was no fat seen in any of the liver sections except in R18 in which a few fine fat droplets were observed in the sinusoids. The failure to produce any toxic effect in rabbits receiving daily infusion for as long as four weeks further strengthens our confidence in the clinical use of the preparation.

SUMMARY AND CONCLUSIONS

Observations were reported on the effects of intravenous administration of a fat emulsion for parenteral nutrition to rabbits. Injection of 1.5 to 3.0 Gm. of fat per kilogram of body weight caused no significant change in arterial blood pressure. The emulsion was not antigenic and did not cause hypersensitiveness. Emulsions which were less than two months old produced no febrile

reaction and the old preparations could be rendered less thermogenic when re-autoclaved. Anemia was not observed after the daily intravenous infusion of 2.0 Gm. fat per kilogram of body weight for as long as four weeks. Slight gain in body weight was demonstrated. Postmortem examinations, microscopic as well as gross, revealed no significant pathologic changes.

It can be concluded that further confidence has been gained in this emulsion for clinical use.

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PRODUCTION OF INSULIN SENSITIVITY WITH THE ADRENOCORTICOLYTIC DRUG DDD (2, 2-bis (PARACHLOROPHENYL)-1,1-DICHLOROETHANE)

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CHAPEL HILL, N. C.

ATROPHY of the adrenal cortex in dogs[1, 2, 3] with conspicuous lesions in the zona fasciculata and reticularis[2] has been reported to occur as a consequence of the oral administration of the compound DDD (2,2-bis (parachlorophenyl)-1, 1-dichloroethane). Inasmuch as the diabetogenic hormones of the adrenal are apparently elaborated in the zona fasciculata,[4] it follows that administration of DDD should provoke increased sensitivity to insulin. The experiments described here were designed to examine this possibility.

MATERIALS AND METHODS

Ten adult, mongrel dogs with weights ranging from 7.7 to 15 kilograms were used. They were apparently in good health and free from external and internal parasites. DDD (200 mg. per day dissolved in corn oil) was given in capsules orally to the experimental dogs as shown in Tables I and II. Control dogs received the same diet of Purina chow and beef scraps as did the experimental dogs.

Intravenous insulin tolerance tests were given to experimental and control dogs according to the schedule shown in Table I. All insulin tolerance tests were done as follows. Food was withdrawn from the cages for forty-eight hours prior to each test. Lilly's Iletin regular insulin (40 units per milliliter) was used, diluted when necessary with distilled water. Venous blood samples were taken before insulin, and one-half, one, two, three, and four hours after insulin injection (Fig. 1). Blood glucose was determined by the method of Folin,[5] using a Klett-Summerson colorimeter.

Three liver function tests were done (Table II). (1) Plasma prothrombin concentrations were done by the modified two-stage method of Ware and Seegers.[6]‡ (2) Serum thymol turbidity determinations were done by the method of Shank and Hoagland[7] using a Klett-Summerson colorimeter with a 660 mμ filter. (3) A modification of Livingston and Bridge's epinephrine test for hepatic glycogenolysis was used.[8] In this test, food was withdrawn from the cages twenty-four hours prior to the procedure. The test dose (0.05 mg. per kilogram) of epinephrine hydrochloride was injected intravenously. Venous blood samples for glucose concentration were taken before epinephrine and one-fourth, one-half, and one hour after epinephrine injection (Table III).

At the time of sacrifice the dogs were killed by intravenous injection of ether and immediately autopsied. The adrenals, pituitary, and samples of the kidney and liver were fixed in ten per cent Formalin, sectioned, and stained with hematoxylin and eosin. Frozen sections were also made of the adrenals and stained with sudan III for demonstration of total lipids.

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TABLE I. DATA ON SIX DOGS GIVEN TWO DOSE LEVELS OF INSULIN DEMONSTRATING THE EFFECT OF ORAL DDD TREATMENT ON INSULIN TOLERANCE CURVES AND CLINICAL BEHAVIOR

DOG	INSULIN TOLERANCE TEST (U. PER KG.)	ABNORMALLY LOW INSULIN TOLERANCE CURVE	CLINICAL EFFECTS
	PRIOR TO FEEDING DDD		
A	0.4	0	0
B	0.4	0	0
C	0.4	0	0
D	0.4	0	0
	3.0	±	0
E	0.4	0	0
	3.0	±	0
F	0.4	0	0
	3.0	±	0
	FED DDD 3 WK.		
A	0.4	+++	Death
B	0.4	0	0
C	0.4	0	0
	FED DDD 5 WK.		
D	3.0	++	Ataxia
E	3.0	++	Ataxia
F	3.0	++	Ataxia
	FED DDD 8 WK.		
B	3.0	++	Coma
C	3.0	++	Ataxia

TABLE II. DATA ON TWO LIVER FUNCTION TESTS: PLASMA PROTHROMBIN CONCENTRATION AND SERUM THYMOL TURBIDITY IN FOUR DOGS TREATED WITH ORAL DDD AND IN A CONTROL DOG

DOG	MEDICATION	PROTHROMBIN CONCENTRATION (% OF NORMAL)	THYMOL TURBIDITY (SHANK-HOAGLAND TURBIDITY UNITS*)	LENGTH OF TREATMENT (WK.)
N	None	100	<5	0
I	DDD	96	<5	5
J	DDD	100	<5	5
K	DDD	103	<5	5
B†	DDD	89	—	8

*Normal range 0-5.
†Plasma stored two months at −60° F.

TABLE III. BLOOD GLUCOSE CURVES OBTAINED AFTER 0.05 MG. PER KILOGRAM EPINEPHRINE HYDROCHLORIDE WAS INJECTED INTRAVENOUSLY INTO CONTROL DOGS AND DOGS FED DDD

The Differences Seen Between the Treated and Untreated Dogs Were Not Statistically Different ($F = 2.28$ With 1 and 16 Degrees of Freedom). However, the Total Number of Six Dogs Used May Be Insufficient to Show Any Difference in View of the Large Animal Variability. Further Observations Are Desirable Using More Dogs and Duplicate Blood Analyses.

DOG	0 MIN.	15 MIN.	30 MIN.	60 MIN.
	CONTROL DOGS			
P	75	125	105	85
Q	60	130	120	105
R	75	120	110	89
	DOGS FED DDD 5 WK.			
I	70	125	110	90
J	62	120	110	100
K	60	120	60	80

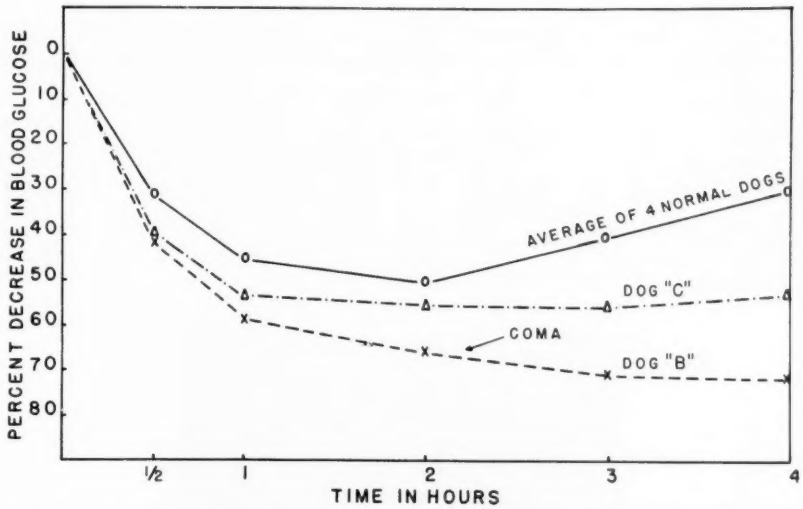

Fig. 1.—Per cent change in blood glucose concentration in four normal dogs and two dogs treated with 200 mg. DDD per day for eight weeks (following the intravenous administration of insulin, 3 units per kilogram).

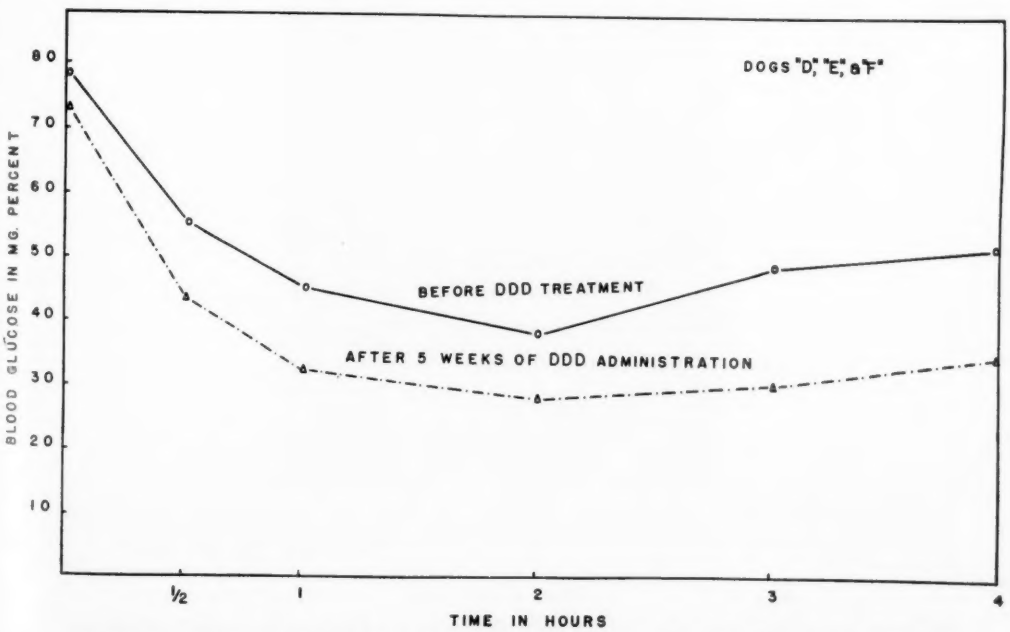

Fig. 2.—Mean data for insulin tolerance tests on three dogs prior to feeding DDD and after five weeks of DDD treatment. It can be seen that the average blood glucose values from insulin tolerance curves done after feeding DDD were lower than when done prior to feeding DDD. This decrease averaged 12 mg. per cent when all blood glucose values in the pre-DDD curves were compared with the post-DDD curves. The decrement was statistically significant, since the probability that it could have arisen by chance alone was considerably less than 1 in 100. In making this test of significance, allowance was made for the variation between and within dogs, and the time-response for the insulin injection. The estimated 95 per cent confidence interval for the average reduction in blood glucose was 7 to 17 mg. per cent.

The hypothesis was also tested that the configuration of the two curves is the same after insulin injection, even though one is at a lower level than the other. This was done by fitting polynomials to the two sets of data portrayed in Fig. 2. It was found that the only difference between the fitted curves was the constant term and that the regression coefficients of blood glucose on time were not significantly different in the two sets of data.

RESULTS

(A) *Insulin Tolerance Tests.*—Three weeks after DDD therapy was begun Dog A developed convulsions and hypoglycemia during an insulin tolerance test (0.4 unit per kilogram), and died after twenty-four hours of continuous tonic-clonic twitching (Table I). Experimental Dogs B and C at this time showed no effect from 0.4 unit per kilogram of insulin, but five weeks later showed abnormally low insulin tolerance curves, transient coma (Dog B), and ataxia (Dog C) when given 3.0 units per kilogram. Controls done at the same time showed none of these manifestations (Fig. 1).

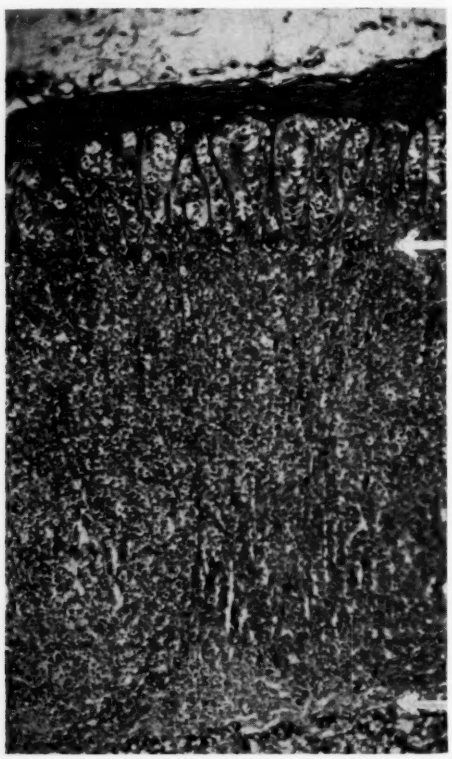

Fig. 3.—Section of adrenal cortex of control Dog N. The glomerulosa is at the top of the photograph, and a narrow strip of medulla can be seen at the bottom. The fasciculata and reticularis extend between the two arrows. (×85. Hematoxylin and eosin stain.)

Dogs D, E, and F received insulin tolerance tests of 3.0 units per kilogram prior to receiving oral DDD, and again five weeks after daily administration of the drug. During the latter test the dogs became ataxic and showed lower insulin tolerance curves when compared with values obtained before DDD therapy (Table I; Fig. 2).

(B) *Liver Function Tests.*—Because DDD is known to cause liver damage[2] and since this in itself may influence the insulin tolerance curve, three tests for functional hepatic insufficiency were made on dogs fed DDD. Table II presents the data obtained when plasma prothrombin concentration and serum thymol

turbidity were determined on such animals. Table III shows data obtained when epinephrine tolerance tests for hepatic glycogenolysis were done on DDD-fed dogs. None of these tests showed significant diminution in liver function.

(C) *Histologic Data.*—The microscopic appearance of the adrenal from control Dog N is shown in Fig. 3. In the dogs treated with DDD, microscopic examination of the adrenals stained with hematoxylin and eosin revealed marked atrophy of the fasciculata and complete atrophy of the reticularis. The glomerulosa remained relatively unchanged (Fig. 4). Dog C, who had an abnormally

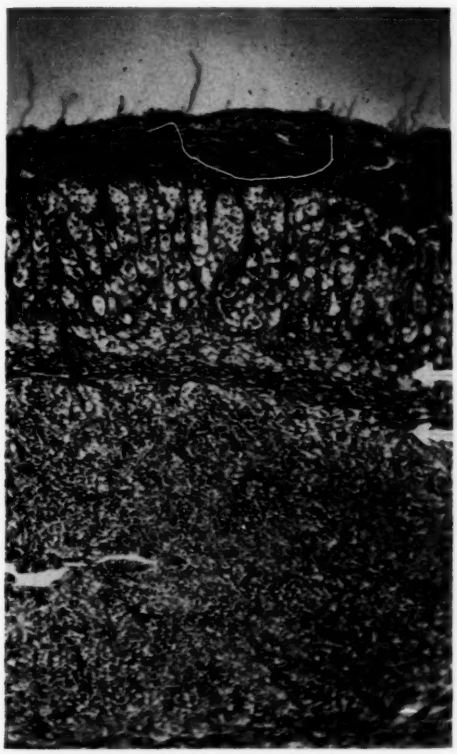

Fig. 4.—Section of adrenal cortex of Dog B photographed at the same magnification as Fig. 3. This dog had been treated with DDD for eight weeks and became comatose during an insulin tolerance test. Note the well-defined glomerulosa which is slightly thicker than that of the control (Fig. 3). The fasciculata and reticularis are almost absent, and a thin band of reticular tissue, measured by the two arrows, is all that remains . The bottom half of the photograph is medulla. (×85. Hematoxylin and eosin stain.)

low insulin tolerance curve but minimal clinical evidence of hypoglycemia, showed less histologically apparent adrenal damage than Dogs A and B who were more sensitive to the insulin. In the dogs treated with DDD the sudanophilic lipids of the atrophic zones adjacent to the medulla were greatly reduced. In the outer cell layers of the fasciculata the lipids were collected in extremely large globules, whereas in the glomerulosa, especially in Dog B, the lipids were present in fine, powdery droplets (Fig. 5). The appearance of lipids in fine droplets has been taken as an indication of increased adrenal activity.[9]

The livers of the dogs treated with DDD showed a moderate degree of central fatty degeneration, as observed by Nelson and Woodard.[2] The kidneys and pituitaries showed no histologic changes.

Fig. 5.—Glomerulosa of DDD-fed Dog B at a higher magnification. A fat stain shows that the glomerulosa is filled with small powdery droplets of lipid (upper half of photograph), and the adjacent atrophic tissue (lower half) is filled with larger droplets. (×185. Sudan III stain. Frozen section 15 μ thick. Green filter.)

DISCUSSION

The production of relative insulin sensitivity in six dogs permits one to draw only preliminary conclusions. However, when taken with the histologic findings in the adrenal cortices of these animals and in light of the newer knowledge of the relation between adrenal cortical structure and function, certain limited comments can be made. For purposes of convenience, comments will be divided into three groups represented by the three principal adrenal cortical hormone types: (1) carbohydrate regulating steroids ("S" hormones), (2) sodium-potassium regulating steroids (Na-K hormones), and (3) androgenic steroids (urinary 17-ketosteroid precursors).

"S" Hormones.—The development of insulin sensitivity in the DDD-treated animals is in accord with the concept of the diabetogenic action of the carbohydrate-active steroids of the adrenal cortex. It is known that adrenalectomy will ameliorate pancreatic diabetes (Long-Lukens animal)[10] and that administration of pure 11-oxygenated adrenal cortical steroids[11] or adrenocorticotrophic hormone

(ACTH)[12] will produce "steroid" diabetes. The glycogenolytic activity of epinephrine from the medulla in insulin tolerance tests was pointed out by Brouha and colleagues.[13] Finally, hypophysectomy with cessation of production of ACTH will ameliorate diabetes (Houssay animal)[14] and will produce atrophic adrenal cortices.[15]

The death of Dog A after only 0.4 unit of insulin per kilogram is noteworthy. Although this dose is in excess of that used clinically in insulin tolerance tests (0.1 unit per kilogram), it is not extreme. Engel and Scott have recently re-emphasized the hazardous nature of the ordinary insulin tolerance test in patients with Addison's disease.[16]

None of the animals in this experiment developed unusually low blood sugar levels after a forty-eight hour fast, whereas the fasting tolerance test has been found useful in confirming the diagnosis of Addison's disease in patients.[17] However, the dogs had practically intact glomerulosas and patients with Addison's disease do not. Recent data indicate that there is some interconversion of desoxycorticosteroids into carbohydrate active steroids in adrenal tissue[18] which might be sufficient to offer protection against fasting in an animal whose fasciculata is completely destroyed. Greep and Deane have recently shown that cortical tissue which has regenerated from the surviving glomerulosa of a gland which has had its medulla and fasciculata curetted out can produce carbohydrate active steroids.[18b]

Evidence points to the fasciculata as the site of production of the carbohydrate active cortical steroids.[4] Nonspecific stresses which elicit endogenous production of ACTH, or administration of ACTH itself, cause an "alarm reaction" in the adrenal cortex of rats and dogs (depletion of lipids) which is most marked in the fasciculata.[4] The administration of corticosterone[4] or cortisone[19] to rats causes atrophy of the fasciculata and reticularis. Adrenal atrophy in the rat caused by cortisone treatment has been shown to be mediated via suppression of pituitary ACTH production.[20]

Na-K Hormones.—There is considerable evidence that the glomerulosa regulates sodium and potassium metabolism. Administration of diets high or low in sodium or potassium cause histochemical changes in this zone.[21-24] Administration of desoxycorticosterone causes atrophy of the glomerulosa.[25] This atrophy can be prevented by maintaining the animal on a low sodium diet.[26] In most animals the glomerulosa remains intact after hypophysectomy while the fasciculata and reticularis atrophy.[15, 27-33] Such hypophysectomized animals remain in electrolyte balance[34-36] although they tend toward hypoglycemia. Daughaday and MacBryde[37] have recently presented additional evidence indicating that the salt-retaining activity of the human adrenal cortex is independent of the pituitary.

The animals in this experiment showed no clinical evidence of lack of the desoxycorticosteroid type of adrenal cortical hormone. On the contrary, one of the animals (Dog B) showed transient periorbital edema. This finding, together with the observation of fine droplets of lipid in the glomerulosa, suggests, if anything, relative hyperactivity of that adrenal cortical region. If there

is a causal relationship between atrophy of the two inner cortical zones and hyperfunction of the outer zone, some light may be thrown on the paradoxical appearance of desoxycorticosteroid effect (hypochloremic alkalosis) in patients receiving cortisone therapy.[38] The effect of cortisone in causing atrophy of the two inner cortical zones was previously mentioned.[19]

From the behavior of the DDD-fed dogs the conclusion appears warranted that in the dog the glomerulosa (not under pituitary ACTH control) is necessary for life, whereas the fasciculata and reticularis (under ACTH control) are not.

Androgenic Hormone.—Clinical and experimental evidence indicates that the reticular zone is involved in the production of adrenal androgenic steroids.[39-41] Since the reticular zone appears to be destroyed in the animals fed DDD, studies of 17-ketosteroid excretion are in order.

Mechanism of Action.—There is no evidence as to how DDD brings about this adrenal cortical atrophy. Finnegan and colleagues[3] have found that the substance accumulates preferentially in body fat, apparently because it is more soluble in fats than in an aqueous medium. These investigators also found it present in relatively high concentration in the adrenal. If DDD exerts a local effect, it is difficult to understand how the zona glomerulosa escapes atrophy since lipids are distributed throughout all parts of the cortex.

Since the zona glomerulosa is apparently independent of the pituitary in rats, dogs, and guinea pigs,[15, 25, 27-33] it should remain intact in the presence of an ACTH blocking agent. The resultant atrophy ought to resemble histologically that obtained by hypophysectomy. This is in fact the appearance in dogs after DDD treatment. No data are available to elucidate the mechanism of this action, although an attractive hypothesis would be that DDD blocks the action of ACTH in the dog.

SUMMARY

Prolonged oral administration of DDD to dogs results in an atrophy of the zona fasciculata and reticularis of the adrenal cortex similar to that obtained by hypophysectomy. Such animals survive, indicating that the two inner zones may not be necessary for the preservation of life. The intact glomerulosa becomes infiltrated with small droplets of lipid material suggestive of a state of increased activity. Dogs so treated show no clinical signs of sodium deficiency and become relatively insulin sensitive. Possible mechanisms of action are discussed.

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STUDIES OF RADIOGALLIUM AS A DIAGNOSTIC AGENT IN BONE TUMORS

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INTRODUCTION

THIS is a report of the first eighteen human subjects in whom radiogallium tracer studies have been made at this medical center.

Laboratory studies of the pharmacology and biochemistry of gallium have shown that this element is selectively localized in osteoid tissues[1, 2] and particularly in centers of osteogenesis.[3, 4]

The purpose of the present study was (a) to develop methods for the localization of Ga^{72} in the human body, by an externally positioned Geiger tube, and (b) to determine the degree of localization of Ga^{72} in patients with neoplastic lesions involving bone. The present study reports results of the study of eighteen cases of primary and secondary bone malignancies.

Gallium is a metallic element having many of the chemical properties of aluminum. The radioactive isotope (Ga^{72}) is a β and γ emitter, of energetic spectrum (max. β 3.1 mev, γ 2.5 mev). This isotope is contained in carrier gallium (0.1 mc. per milligram Ga) and administered as the citrate.[5]

LABORATORY METHODS

Counting Techniques.—There are many factors which must be considered when attempting to localize in the human body, by external counting methods, a particular organ or structure which contains a quantity of some radioactive substance. The principal factors are (1) volume of tissue containing a high concentration of the isotope, (2) differential distribution of the isotope between the localized area and the adjacent tissues, (3) depth of center of mass, i.e., distance to counter tube, (4) degree of collimation obtainable with jacketed Geiger tubes, (5) the nature of the radiation and its absorption characteristics in tissues, and (6) absorption characteristics between counter and source.

In order to study the effectiveness of (I^{131}) radioiodine diagnostic and therapeutic procedures, various end-window Geiger tubes were fitted with lead shells. This was an attempt to produce a collimated beam of gamma radiation which would more closely define areas of active thyroid hyperplasia. Considerable success was obtained in these studies.[6] It was found that the same general type of jacket was satisfactory for such studies of Ga^{72}, but a considerably larger and denser shield was needed in order to produce a sharply collimated beam from the skin surface of a patient receiving Ga^{72}. In Fig. 1 is presented a sketch of the Geiger tube and the surrounding jacket (weight 7 kilograms) as used in these studies.

In Fig. 2 are presented graphically results of a study of the collimating ability of the tube and jacket described in Fig. 1. This study was made using a 2 cm. planchet of Ga^{72}. The "theoretical cutoff zone" is the geometric projection of lines passing through the edges of the tube window and the shield port. This zone delineates the area beneath the tube which emits radiation directly (disregarding scattered radiation) into the tube.

The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or endorsement of the Navy Department.

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The "line of 50 per cent cutoff" is the *observed* lateral distance at which the counting rate is reduced to one-half the maximum observed if the sample was placed opposite the shield port.

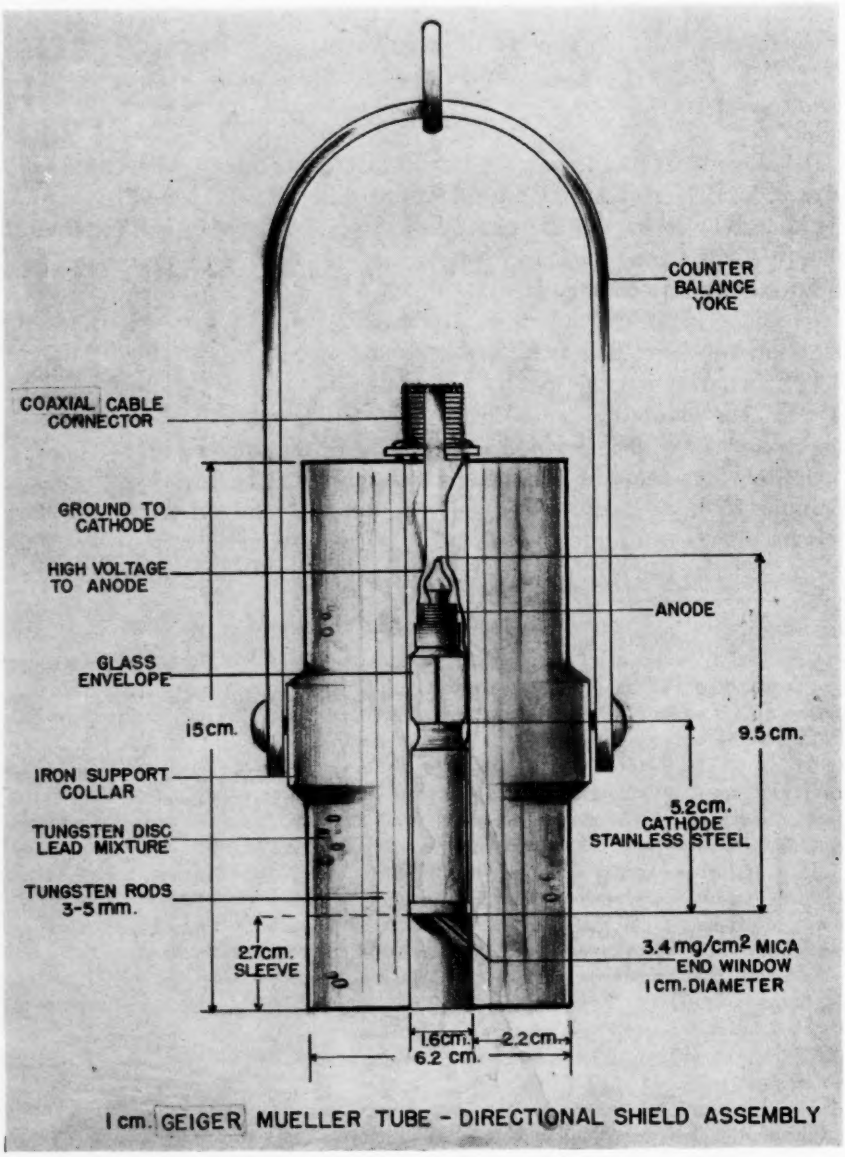


Fig. 1.

The lines denoting the relative counts per minute indicate the counts observed as the target was moved laterally in front of the tube at some definite distance (tube to target distance) i.e., 40, 90, 120, or 150 millimeters.

Study of Ga^{72} in Phantom.—The purpose of this phase of the investigation was to study the methods which were being used clinically and to evaluate their significance under controlled laboratory conditions.

A simulated human torso was made by filling a wooden box (60 cm. by 52 cm. by 24 cm.) with hulled rice (sp. gr. 0.825). To the rice was added, by scattering through the mass, a water solution of Ga^{72}. The rice was then thoroughly mixed. A concentration of 0.07 to 0.08 μc Ga^{72} per liter of rice gave a count of 1,200 to 1,500 counts per minute at a distance of 2 cm. from the surface. This approximates what had been obtained in surface counts of human beings who had been administered 300 to 400 μc Ga^{72} intravenously. The jacketed Geiger tube shown in Fig. 1 was used throughout these phantom studies.

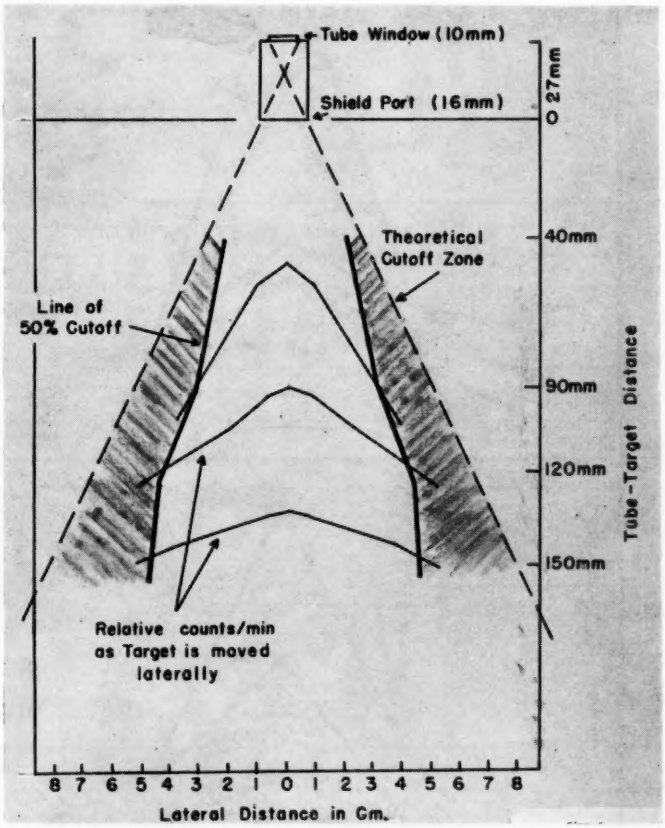


Fig. 2.

In order to give reproducible geometry, a steel rail arrangement was made so that the placement of the Geiger tube relative to the surface and center of the mass of the "phantom" was constant. A series of volumetric flasks from 10 c.c. to 2,000 c.c. were filled with a water solution having a concentration of Ga^{72} (microcuries per liter of water) from two to forty times the concentration of Ga^{72} (microcuries per liter of rice) contained in the phantom. These flasks were prepared in order to simulate organs or structures of different sizes and having different concentrations of Ga^{72}.

In making a study of the effect of the counts obtainable at the surface of the rice, the Geiger tube was successively placed at each of the five counting points (Fig. 3). When a satisfactory base line had been obtained, one of the flasks was buried in the rice beneath the center point. In each series the center of the mass of the flasks was located at some predetermined distance below the surface of the rice (4 or 9 cm.). Counts were taken at

all five points across the surface of the rice after burying the flasks. In Fig. 3 is shown a graphic representation of the counts obtainable with various flasks containing Ga^{72} concentrations five and twenty times the concentration of Ga^{72} in the body of the phantom. These findings are typical of the results obtained at other concentrations.

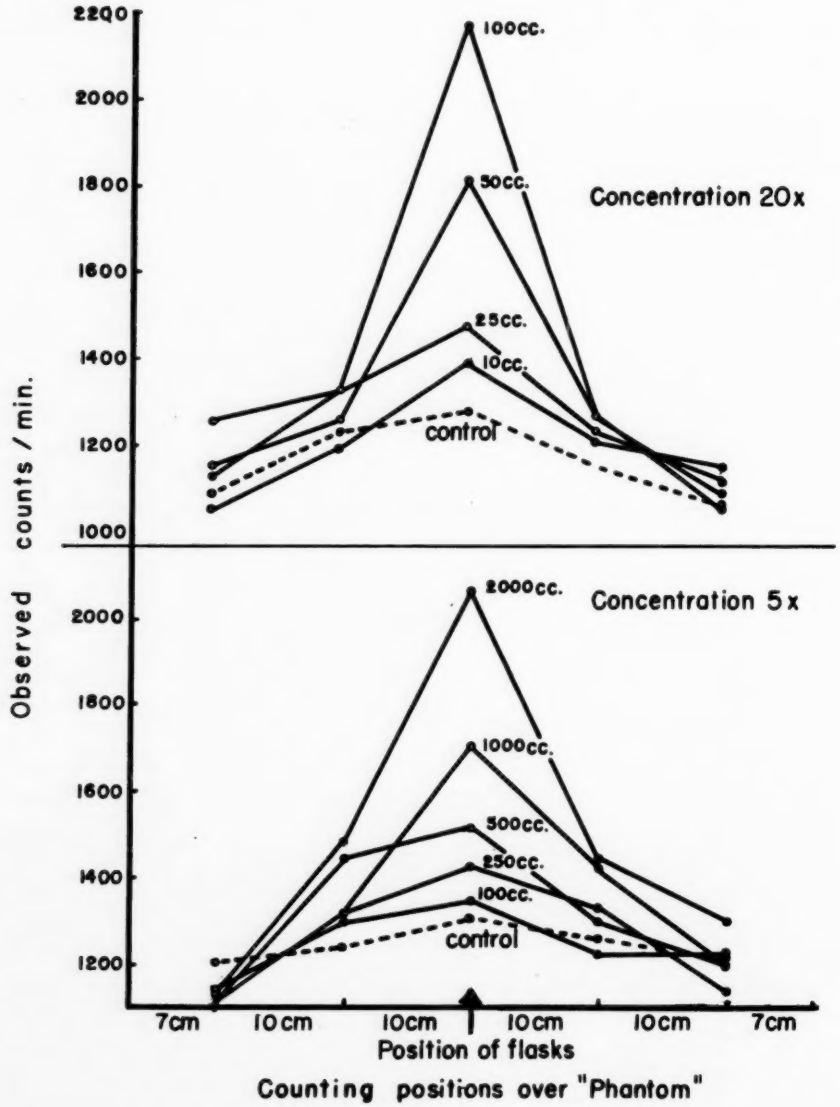


Fig. 3.—Influence of the size of the flask, the concentration of Ga^{72} therein, and position of the Geiger-Müller tube on the observed counting rate.

In Fig. 4 is shown graphically a résumé of the results of this phase of the study. In this figure is plotted the relative percentages of counts obtainable at the center point of the phantom plotted against the diameter of the flask containing various concentrations of Ga^{72}. These findings indicate the counts obtainable at the surface are a function of

both the size of the buried flask and the concentration of the Ga^{72} contained therein. There are factors in these findings which limit their usefulness, particularly in the smaller-sized flasks or at the lower concentrations of Ga^{72}. When the counts over a buried flask do not exceed the background by more than 5 per cent, the significance of the result is questionable.

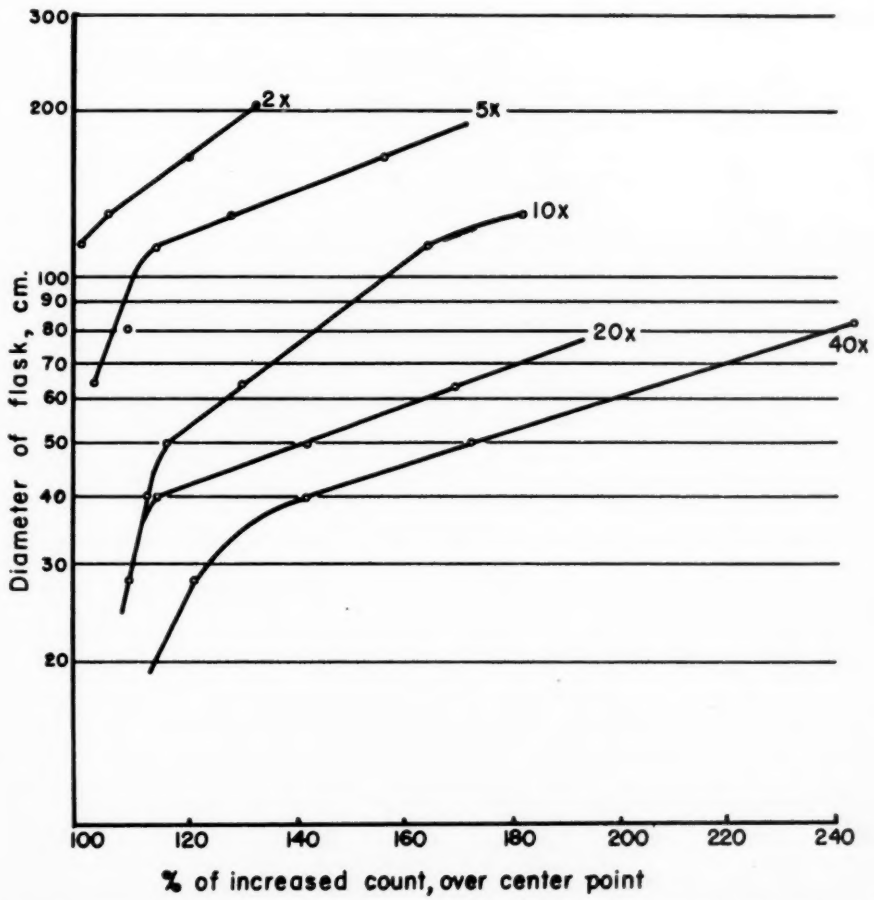


Fig. 4.—Relationship of the diameter of the flask, the concentration of the Ga^{72} therein, and the increased count obtained over these flasks in the phantom.

To approximate the concentration or degree of localization in diagnostic studies with Ga^{72}, the findings shown in Fig. 4 are of significant aid. The diameter and depth of the lesion can be estimated from x-ray films. By external counts over the body, using the Geiger tube described in Fig. 1, a series of counts will be obtained. If one determines the percentage of count over the lesion to be, for example, 115 per cent of the count over adjacent tissues and the diameter of the lesion to be 8 cm., at a depth of 4 cm., then, as an approximation, the concentration of Ga^{72} in the lesion is five times that in the surrounding tissue. If the diameter of the lesion is 2 cm., at a depth of 9 cm., and the observed count is 125 per cent of the general average, the concentration of Ga^{72} in the lesion approximates forty times the general body concentration. Within limits the curves shown in Fig. 4 may be used to approximate the concentration in various lesions in human beings, in cases of malignancies.

It is realized this indirect method of determining concentration of a chemical substance in a specific location in the human body is only an approximation. It is, however, based on sound physical principles and seems to be the only means, short of biopsy, of estimating the concentration of this isotope in osteoid structures. More significantly for diagnostic procedures, these laboratory findings indicate that an increase of 10 to 20 per cent of counts over a localized area is evidence of marked localization of Ga^{72} in some structure beneath the surface.

CLINICAL METHODS

Selection of Cases.—The patients were primarily selected from those with proved malignancies, having x-ray evidence of new bone production, or metastatic lesions in bone. Later it was found that patients having localizing bone symptoms but without x-ray confirmation were also suitable for these diagnostic studies.

Administration of Ga^{72}.—(Radiogallium (Ga^{72}) was administered as a citrate complex (300 to 400 μc) accompanied by 3.8 to 5.0 mg. carrier gallium, contained in 1 c.c. of solution.[5] The Ga^{72} citrate solution was measured in a lead-shielded tuberculin syringe[7] and injected into the rubber tubing of an intravenous infusion drip apparatus. The antecubital vein of the arm was used as the point of administration. The injection was made in one minute. No adverse local or generalized reactions have been observed in any case to date.

Surface Counting.—Suitable points on the body of the patient were marked with indelible ink. Posteriorly, these included twelve points, every 5 cm. along the spine and two series of points laterally over the pelvis. Anteriorly points were marked as follows: two rows of six points, from each groin superiorly to the second intercostal space. Two additional points were located on the upper and lower points of the sternum.

The Geiger tube (Fig. 1) was localized over each point at the surface of the body and a one-minute count recorded. By counterbalancing the jacketed tube, it was possible to maintain the end of the shield gently pressed against the skin surface. No undue discomfort to the patients resulted.

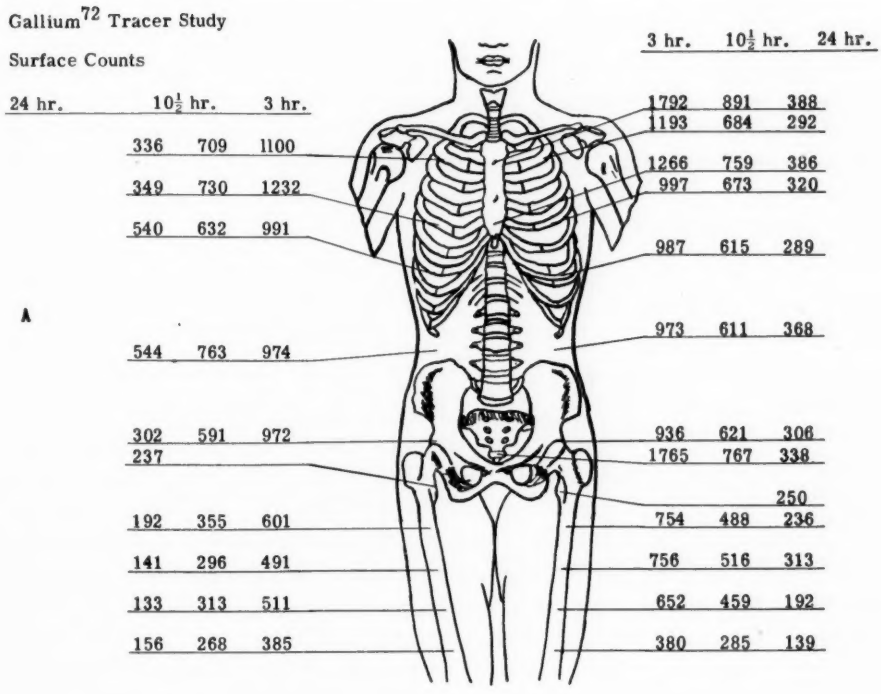
The pattern of counting areas included forty points. Time of each series of counts required approximately one hour. Counts were made at three, ten, and twenty-four hours after the intravenous injection of the tracer dose of Ga^{72} (300 to 400 μc). With this dose it was possible to obtain counts of 600 to 1,100 per minute at the skin surface of the patient three hours after administration of the Ga^{72}. The background rate of the tube used is 7 to 9 counts per minute. In Fig. 5 are shown details of the results of a routine examination of a patient by this method (Case 8).

The addition of counts, taken directly over the xyphoid process at 30 cm. and 90 cm. distances, each count for three minutes, was made in the last five cases of the series. This would tend to indicate the rate of concentration change in the trunk of the patient and act as a base line for comparison of concentrations within each localized area. The biologic half-life as determined by the method in the human patient is 12 to 13 hours.

A significant aid in determining the areas of localization of gallium in various tissues has been the determination of the relative increase of Ga^{72} during the three hour to twenty-four hour period after intravenous injection of the gallium citrate.

The observed Geiger-Müller count over a certain point at three hours after injection is taken as a reference value. The counts over this exact point at ten hours and twenty-four hours, when corrected for decay to the three hour time ($T_{½}$ = 14.3 hr.), will indicate whether there has been an increased deposition of gallium in the structures beneath the counting point.

In many normal tissues the amount of gallium will decrease, as indicated by the foregoing calculations. However, in many areas of bone involvement there is a marked increase in deposition of gallium, as indicated by the external counts. This fact has proved most significant in these diagnostic studies.



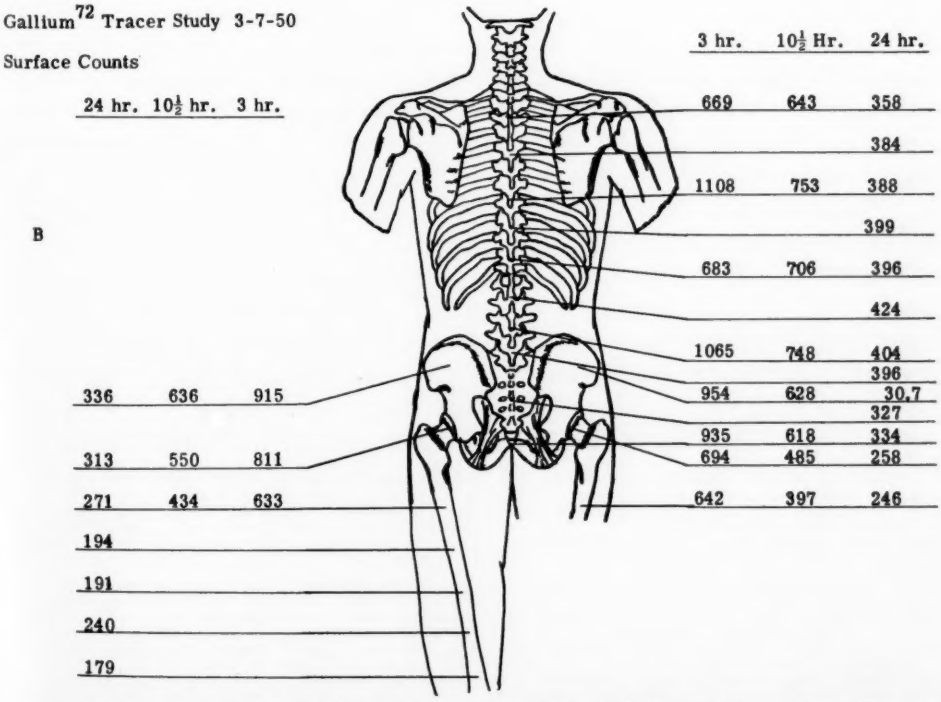


Fig. 5.—*A*, Anterior of body surface. *B*, Posterior of body surface. The pattern and record of the Geiger-Müller counter rates per minute as made in Case 8. The time period of each count series is from the time of intravenous injection of the tracer dose.

RESULTS

Case Histories.—In Table I are shown the clinical findings and histories of the first eighteen patients, during the first seven months of the use of Ga^{72}. Of this group, fifteen resulted in positive localization of significant metastatic or primary bone tumor areas, two gave negative results which are consistent with the limited clinical follow-up to date, and in one case a small osteolytic lesion of the ischial spine was missed.

Osteogenic Lesions.—One primary bone tumor is included in the series, an osteochondrosarcoma. This case (Case 7), in a 39-year-old man, had been followed clinically for a period of more than eight years. During this time the patient had had three complete resections of the tumor and two courses of deep x-ray therapy.*

Case 6 was that of a malignant degeneration of an area of myositis ossificans with a locally infiltrating osteogenic sarcoma. This young man, who is now 29 years of age, had received a course of prophylactic deep x-ray therapy for carcinoma of the testes. The areas of treatment included three over the lower abdomen and two areas in the lower half of the spine. The malignant degeneration apparently originated in the upper of the two posterior areas. Following a biopsy of the lesion, the skin failed to heal and at the time of the tracer study there was a large (15 cm.), friable, bleeding, open area. For this reason, it was difficult to get exact counts over the different nodules and to position the patient as much as desired. The standard pattern of counting was modified. All palpable nodules gave significantly increased counting rates. A single isolated lesion on the anterior chest wall could be well studied and indicated a concentration of Ga^{72} of fifteen times that of the surrounding apparently normal tissue. This finding agrees well with what has been found in the other cases.

The remaining cases of the osteogenic group consist of five carcinomas of the prostate. In general, these cases were collected as the original choice for study because the osteogenic nature of the lesion would make them most comparable to growing bone as determined from the animal studies. Significant localization was found in each of these five cases to be proportional to the lesions as indicated by x-ray examination. It was noted in Case 4 that while at the time of the study only one lesion had been localized by the tracer studies, follow-up x-ray films taken one month after the tracer study showed a beginning bone change in each of the two areas; the smaller of these two areas, in the ilium, was approximately 3 cm. in diameter. Case 5, that of a 60-year-old man, had been followed for approximately four years as a fairly typical

*The amount of bone proliferation as compared with the cartilage contained in the tumor mass was relatively small. Areas of increased G.M. counting rates were confined to these areas of bone production and were significantly higher than the counting rates of adjacent areas overlying cartilage. The general level of counting over the palpable tumor was lower than over the opposite area presumed to be normal. The repeated operations and two courses of x-ray therapy may have interfered with the blood supply to the area and prevented absorption and concentration. This is the only case in the series to date in which the counting rate over an area of a clinically suspected lesion or an area of x-ray change was decreased below the adjacent or opposite normal area.

Paget's disease. Two years ago a positive diagnosis of carcinoma of the prostate was made. At the time of this study the Paget's disease, which had been localized in the right pelvis and right femur, had changed very little. Scattered areas of well-circumscribed osteogenic lesions, 1 to 2 cm. in diameter, were found in the wings of both ilia and in the upper portion of the shaft of the left femur. It is believed on the basis of the prostatic tumor that this represents a separate condition of bone involvement of carcinoma of the prostate. The radiogallium seemed to localize and concentrate equally well in the lesions of both the Paget's disease and the carcinoma of the prostate.

Osteolytic Lesions.—These eleven cases are all secondary metastatic lesions or strongly suspected metastasis in the presence of known carcinoma. The first case of this group which was studied was Case 8, a bronchogenic carcinoma. This patient had developed a large soft tissue tumor associated with an area of destruction of the proximal shaft of the left femur. The patient was approximately one year postoperative for the chest lesion and there was no evidence of a local recurrence. It was considered that if no other areas of metastasis could be localized, and none had been found by x-ray film study, the treatment of the area in the left femur might be more definitive and complete. Concentration and localization within the known area of metastasis was equally as good as in the most striking of the osteogenic lesions. The area went on to pathologic fracture, but in the past four months no evidence of other bony metastases have been found. The localization as found in this case encouraged us to try other osteolytic lesions.

Case 9 was that of a 55-year-old man with gastric carcinoma. This patient had progressed from a symptom-free stage to that of an inoperable carcinoma of the stomach within three months, and within two months after the operative procedure, a total of five months, had at least four separate areas of metastases to the spine. The general progress of the patient was rapidly downhill, and there was marked jaundice as evidence of liver metastasis. Radiogallium Ga^{72} as concentrated in the liver was the lowest of any case of this series. The tracer study localized the known lesions well and in addition indicated possible involvement in the pelvis.

Case 16, a 54-year-old white woman with carcinoma of the breast, had evidence of widespread bony metastasis at the time of her original operation more than one year ago. Hormone therapy with testosterone given over a period of approximately nine months had changed the bone lesions from markedly osteolytic to a significant osteoblastic quality. The hormone therapy was causing a considerable amount of nausea, vomiting, and other generalized symptoms. The bone pain as a result of this therapy had completely disappeared and the patient experienced only a tired feeling after doing a considerable amount of housework. This therapy was therefore discontinued. In the following three months, the x-ray findings were seen to change from the osteoblastic quality into almost the original osteolytic quality. Because of the far-advanced nature of the bone lesions, it was desired to use the Ga^{72} tracer study as a method of determining areas of possible pathologic fracture. The

TABLE 1

CASE	AGE AND SEX	DIAGNOSIS*	EXTENT OF LESION	SIGNIFICANT CONCENTRATION† OF Ga^{72}	24-HOUR URINARY EXCRETION OF Ga^{72} (%)	CONFIRMATORY EVIDENCE AND REMARKS
			Osteoblastic Lesions			
1 W. L. F. 11/29/49	49–M	Ca. prostate—P	X-ray—Progressive metastasis: Spine—Bodies of thoracic and lumbar Pelvis—Bilateral widespread Femurs—Proximal ends, most on right	Spine—Proportional to x-ray changes Pelvis—Most in area of greatest osteoblastic activity Femurs—Significant localization	6.9	Concentrations were so definite that differential counts from normal to involved bone areas with even partially shielded and nonshielded G. M. tubes gave significant results
2 S. G. S. 12/28/49	69–M	Ca. prostate—P Adenocarcinoma	X-ray—Generalized metastases Thoracolumbar spine Pelvis	Spine—Proportional to x-ray changes Pelvis—Generalized increased activity. Left ilium increased over right. Conc. 8 ×	6.6	Follow-up x-ray studies Patient discharged to home on stilbestrol therapy
3 W. L. M. 1/17/50	49–M	Ca. prostate—P	X-ray—Generalized metastases Thoracolumbar spine Pelvis All bones, even ribs and extremities showed mixed destructive and osteoblastic changes	Generalized throughout bone structures, all bony prominences had an increased counting rate Spine and pelvis most, proportional to the x-ray film changes	6.3	Follow-up x-ray studies. Post mortem
4 J. P.	82–M	Ca. prostate—P	X-ray—Spine—localized Collapse of L-2	Spine—Only area of concentration centered over L-2 Increased Conc. 10 × Pelvis—Right ilium Femur—Left proximal shaft Increased Conc. 30 ×	5.8	Lesions confirmed by x-ray films one month following tracer study Post mortem
5 E. R. 1/24-50	60–M	Ca. prostate—P Paget's disease of right ilium X-C	X-ray—Pelvis—localized Expanding lesion right ilium Circumscribed areas both ilia Right femur—Expanding mixed lesion Left femur—Circumscribed areas	Pelvis—Generalized increased activity Right ilium increased over left Conc. 16 × Femurs—Both increased Righ increased moderately more	11.2	Follow-up x-ray studies
6	29–M	Osteogenic sar-	X-ray—Trabecular bone identified			

6 M. H. 12/20/49	29–M	Osteogenic sarcoma—P Malignant degeneration of myositis ossificans following deep x-ray therapy for Ca—testicle	X-ray—Trabecular bone identified in several of the larger subcutaneous nodules General—Widespread subcutaneous nodules over mid-back and spreading around to left chest	Back—All nodules showed increased concentration Chest—Localized 6 cm. nodule Conc. 15 ×	11.0	Areas as palpated
7 C. F. M. 1/10/50	39–M	Osteochondrosarcoma Recurrent—P	X-ray—Right ilium with extension into left pelvis and right flank	Small areas within extent of lesion gave as much as Conc. 5 × as compared with adjacent areas	13.5	Distribution consistent with pattern of cartilage and bone proliferation
			Osteolytic Lesions			
8 L. S. 3/7/50	62–M	Bronchiogenic Ca.—P Epidermoid carcinoma	X-ray—Destructive lesion of the proximal shaft of the left femur, 3 × 5 cm., associated with palpable soft tissue mass	Left femur—Limits of destructive lesion indicated by pattern of counts. Conc. 40 × Pelvis—Hip area significantly high	3.6	Known lesion well localized No clinical evidence of other bone metastasis in 4 mo.
9 W. H. 4/4/50	55–M	Gastric Ca.—P	X-ray—Spine—Multiple areas of destruction including: C-6, T-6, T-10, T-11 and L-5	Spine—Areas as noted on x-ray films C-6 Conc. 8 × T-10 Conc. 8 × L-5 and S-1 Conc. 7 × Right ilium—Anterior and posterior localization Conc. 8 ×	24.8	Known lesions well localized Right ilium confirmed at post mortem
10 W. H. H. 4/11/50	64–M	Abdominal mass 15 cm. in diameter. No indication of exact diagnosis after G.I., B.E., or I.V.P. studies X-C	X-ray—No changes at time of study Bone pain—Acute and increasingly severe pain and aching over L-3 Deep tenderness—localized within a small area of L-3	Spine—Lumbar area, centering over L-3 extending from L-1-L-5 Conc. 12×	16.5	X-ray—Lesion of destruction was identified 1 mo. after tracer study in the body of L-3 X-ray therapy—Given immediately after tracer study to lumbar spine gave very satisfactory palliative relief
11 I. P. 4/18/50	28–F	Ca. breast—P	Spine—Bone pain and deep tenderness proximal lumbar region Atonic bladder and bowel X-ray—No bone lesion	Spine—Increased and localized concentration over L-1, L-2, L-3 Conc. 15 × Pelvis—Right mid-section Conc. 8 ×	20.0	X-ray therapy—Moderate and significant palliative response to deep x-ray therapy over lumbar spine
12 F. B. 4/25/50	45–F	Ca. breast—P Adenocarcinoma	X-ray—No bone lesions seen on repeated series Bone pain—Lower back and left hip 2 yr., increased frequency and more localized previous 3 mo.	None	33.0	No change in clinical picture in past 2 months

(Continued on following page.)

TABLE I—CONT'D

CASE	AGE AND SEX	DIAGNOSIS*	EXTENT OF LESION	SIGNIFICANT CONCENTRATION OF Ga^{72}	24-HOUR URINARY EXCRETION OF Ga^{72} (%)	CONFIRMATORY EVIDENCE AND REMARKS
13 W. L. 5/2/50	45–M	Ca. of bladder—P Transitional cell Grade III	X-ray—No lesions found Bone pain—increasing pain in the lower spine and left hip Local tenderness—in lower thoracic, mid-lumbar and upper sacral areas	Spine—Localization at: T-8, T-9, T-10 Conc. 7 × L-1, L-2, L-3 Conc. 8 × S-1, S-2 Conc. 7 × Pelvis—Some increased concentration, more on the left	10.7	X-ray therapy—Significant relief of pain after palliative treatment to lumbar spine
14 J. A. 5/16/50	48–M	Ca. of lip—P Squamous cell Grade III	X-ray—Left ischial tuberosity Bone pain—Spine and pelvis Soft tissue extension to neck and shoulders with generalized subcutaneous nodules	None	23.5	Known area by x-ray too close to bladder for localization
15 C. S. W. 5/23/50	49–M	Multiple myeloma—P 50% replacement of sternal marrow	X-ray—Generalized, medium sized lesions in skull, ribs, spine, pelvis, and femurs. Collapse of body of T-10. Lesions progressive on serial films	Spine—Generalized increase of concentration, most over T-10, T-11 Conc. 10 × L-2, L-3 Conc. 8 × Pelvis—High area over right iliac crest	9.7	Consistent with degree of involvement as seen on x-ray films Highest concentration over area of collapsed vertebral body
16 M. V. 6/1/50	54–F	Ca. breast—P Carcinoma simplex	X-ray—Generalized, moth-eaten, most prominent over: Lumbar spine Pelvis Mixed osteoblastic-osteolytic lesions	Increased concentration within all bone areas studied, most in Spine—lumbar Conc. 7 × Pelvis—right post Conc. 5 × Femurs—both increased, left most	15.9	X-ray changes in spine and pelvis consistent with localization Concentration in femurs significant as compared with minimal x-ray findings
17 N. J. A. 6/13/50	39–F	Ca. thyroid—P Papillary adenoma	X-ray—Area of rarefaction left humerus Bone pain—localized areas with deep tenderness over: Left humerus Spine L-3 Right femur—mid-shaft	None	13.9	X-ray opinion modified after this study, to have serial films as follow-up Negative I^{131} tracer study for functioning thyroid except in the neck region
18 E. E. G. 6/20/50	62–M	Ca. of bladder—P Transitional cell Grade III, recurrent	X-ray—Destruction of articular facets of right side of L-2 and L-3 Bone pain—Lumbar spine with radiation into right leg	Spine T-8 and T-9 Conc. 7 × L-2 and L-3 Conc. 9 × Pelvis—Bilaterally, front and back, most just superior to acetabula Femurs—Possible bilateral involvement	17.1	Known lesion highest concentration, other areas had good comparable amounts

*Diagnosis: P, pathologic sections; X, x-ray impression; C, clinical impression.

†Concentration: Estimated in terms of the apparent concentration in the lesion as compared with an uninvolved area of bone opposite or adjacent. In reference to the spine, the counting rate for an area is compared with the average of all points on the spine. Conc. 8× means a concentration of eightfold greater than the bone area used for comparison, etc.

counting rate over all of the bony prominences was increased, with significant concentration within the lower spine and pelvis. Since the gallium study, the patient has had an increasing amount of bone pain to the extent that gallium therapy has been considered. The hormone therapy has been reinstituted in an effort to enhance the concentration of the radiogallium.

Included with the osteolytic lesions is a single case of multiple myeloma. This patient, Case 15, a 49-year-old white man, had a very recently diagnosed multiple myeloma. Although the lesions were of moderate size and widespread, there was only one area of collapse within the spine. A sternal marrow count showed approximately 50 per cent replacement with typical plasma cells and multinucleated plasma cells. Palliative x-ray therapy had been given over the area of the known collapse in the region of T_{10}. This had resulted in significant relief of bone pain. It therefore seemed consistent that an isotope was the logical means of therapy. The first choice was radiophosphorus (P^{32}). A tracer study with radiogallium was done at this time in order to avoid a delay in starting radiogallium therapy in case the radiophosphorous therapy was unsatisfactory. The results of this study were quite comparable to those seen in Case 3 of carcinoma of the prostate with generalized metastases and Case 16 of carcinoma of the breast. In the five-week follow-up period following P^{32} therapy, a general improvement of the patient has been sufficient so that to date it has not been necessary to consider any other type of therapy.

Urinary Excretion.—The urinary excretion in these eighteen patients has been studied in considerable detail. Each separate urine specimen voided by the patient during the first twenty-four hours of the study was radiometrically measured. In general the excretion pattern is similar to that of radioiodine I^{131} [6, 8]; the principal amount excreted is within the first six hours after the dose. The rate of excretion rapidly drops after that period and is negligible after twenty-four hours. From Table I it can be seen readily that in carcinoma of the prostate the amount of excretion is remarkably small. Osteogenic lesions tend to retain more of the total amount of the tracer dose than osteolytic lesions. In the majority of the cases, the amount of Ga^{72} excreted is inversely proportional to the extent of bone involvement. However, in a significant number of cases the pattern and amount of Ga^{72} excretion differ from the rest of the findings.

Additional study of these differences indicated that a tracer dose of 300 to 500 μc of Ga^{72} is sufficient to get reasonably accurate counting rates during the time of measurement of the urine activity. The radiometric method has been checked in a number of cases against the total amount of carrier gallium as excreted in the urine and determined by chemical methods.[1] The agreement between the two methods is sufficient to indicate the practical accuracy of the radiometric method.

The frequency and the total amount of urine voided seem to have an effect on Ga^{72} excretion. In relative polyuria with amounts of urine over 3,000 c.c. in twenty-four hours, there tends to be an increased excretion of gallium.

*Diagnosis: P, pathologic sections; X, X-ray; Conc. 8× means concentration of eightfold greater than the bone area used for comparison, etc.

†Concentration: Estimated in terms of the apparent concentration in the lesion as compared with an uninvolved or adjacent area as compared with the average of all points on the spine. In reference to the spine, the counting rate for an area is compared with the average of all points on the spine.

SUMMARY AND CONCLUSIONS

Geiger counting techniques applied to the skin surface of human beings have been shown to be useful for the localization of radiogallium (Ga^{72}) citrate in osteoid structures.

Intravenous tracer doses of Ga^{72} citrate have been selectively concentrated in the osteoid lesions, both osteogenic and osteolytic, in fifteen of eighteen cases of primary and secondary bone malignancies.

Concentration of Ga^{72} in malignancies involving bone approaches twenty times that found in the adjacent bone.

Early metastases to bone have been identified through the use of tracer amounts of Ga^{72} before changes could be identified by x-ray film study.

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CRYOGLOBULINEMIA

III. Influence of a Cryoglobulin on the Suspension Stability and Sedimentation Rate of Erythrocytes

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THE stability of erythrocytes in suspension in plasma or other fluids depends upon their degree of aggregation and upon the colloidal state of the fluid in which they are suspended. In serum and plasma, the concentration of fibrinogen or its components and of globulins has been found to be especially influential but the effect of individual proteins is only partially understood. The isolation in our laboratory of a chemically pure, apparently homogeneous cryoprotein[1] from a patient with myeloma[2] offered an unusually favorable opportunity to evaluate the influence of a single factor upon the suspension stability and sedimentation rate of red blood cells.

The patient from whom the protein was obtained was a man 58 years of age who suffered from sensitivity to the cold, Raynaud's phenomenon, bleeding from mucous membranes, retinal hemorrhages, progressive deafness, and arthritis, a combination of symptoms which were thought to be attributable at least in part to the presence in his circulating blood of a cold precipitable protein in the remarkably high concentrations of 5.4 to 9.8 Gm. per cent.

OBSERVATIONS

Gross alteration in the suspension stability of erythrocytes characterized the blood of this patient. Indeed, it was the abnormal behavior of his blood in the performance of routine examinations which provided an early clue to the diagnosis of multiple myeloma and cryoglobulinemia.

Venipuncture was easily performed but withdrawal of blood through routine needles of size 20 was difficult because of rapid clogging. It was found necessary to warm the syringe to 50 to 60° C. and to use a No. 18 needle. The blood collected under these circumstances was discharged into a receptacle in a water bath at 37 to 40° C. and allowed to clot at this temperature. Subsequently until serum was separated the blood was maintained at 37° C. Blood withdrawn directly into pipettes for red blood counts, hemoglobin, etc., was frequently found to "jell," thus preventing the usual technical procedures or impairing their accuracy.

Striking abnormalities were noted in the behavior of blood collected in the presence of anticoagulants. Although with different anticoagulants these varied in degree and in the time interval before development, the pattern was always similar. At room temperature there could be noted, within ten minutes

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after collection in potassium oxalate or Heller-Paul mixture, a marked sedimentation of the cellular elements in the collection bottle with opalescent grayish-white particulate material as precipitate which could be distributed over the sides of the bottle by shaking. Agglutination of the formed elements gave an appearance of "red sand." Similar behavior was observed with heparin or oxalate as the anticoagulant, and in defibrinated blood. With sodium citrate at room temperature the appearance of the phenomenon was delayed for a period as long as one hour and therefore permitted observation of blood sedimentation. When blood containing sodium citrate was maintained after withdrawal at 37° C., the macroscopic appearance of agglutination and opalescent precipitation did not occur during the period of observation.

In oxalated blood a small amount of precipitate could be observed even at 37° C. and on centrifugation at this temperature appeared in varying amounts between the layers of plasma and red blood cells. This was observed consistently when hematocrit tubes were spun in a large 37° C. incubator by a centrifuge which varied slightly in temperature during the procedure. In bottles of oxalated blood maintained at 37° C. there was rapid sedimentation of the cells with grossly homogeneous supernatant plasma. In many instances the rate of gross sedimentation was so rapid in the blood bottles that the phases of aggregation, rapid sedimentation, and packing could be followed clearly in the course of twenty to thirty minutes. These observations suggested the following studies.

A. Gross and Microscopic Appearance of Oxalated Blood at Room Temperature.—Blood was collected in potassium oxalate anticoagulant (10 mg. to 10 c.c. blood). At 26° C. there was gross alteration of the physical character which on first inspection resembled autohemagglutination but on further observation was shown to be quite dissimilar.

Opportunity was presented to compare the characteristics of this blood with those of a patient who had developed the phenomenon of cold agglutination and autohemagglutination from an unknown cause and in the extraordinary titer of 1:1,000,000. No cryoprotein could be demonstrated in her plasma. Microscopic and photographic examination revealed that the phenomena of aggregation of red blood cells were entirely different in the two instances. The differences are shown in Fig. 1.

The abnormality in the blood of the patient with cold agglutinins but no cryoglobulins was easily identified as autohemagglutination with diffuse clumping of red cells and no precipitate formation. In the patient with cryoglobulinemia but no cold agglutinins, the apparent agglutination or "red sand" appearance at room temperature was shown to be due to the precipitation of cryoglobulin with physical enmeshing of red cells and mechanical approximation of red cells by the precipitate and was regarded as *pseudoagglutination.* Under the microscope at room temperature three distinct physical states were observed to occur in the oxalated blood from the patient with cryoglobulinemia. (1) Aggregates of red cells in the midst of the cryoglobulin precipitate, (2) rouleaux formation in juxtaposition to the masses of cryoglobulin precipitate, and (3)

normally distributed cells at variable distances from the area of cryoglobulin precipitate. On warming the slide to body temperature and above, there was loss of the appearance of agglutination and disappearance of the cryoglobulin precipitate.

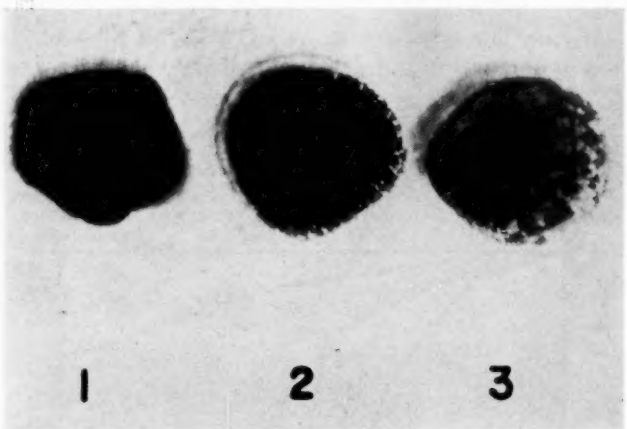

Fig. 1.—Comparison of drops of blood with Heller-Paul solution as an anticoagulant. *1*. Normal. *2*. Blood with cold agglutination titer of 1:1,000,000. *3*. Blood containing cryoglobulin.

B. The Effect of Dilution on the Gross Appearance of the Blood.—Oxalated blood from our patient was diluted with normal saline and observed on a slide for the characteristic "red sand" appearance.

1. With 1:2 dilution the appearance of precipitate formation and red blood cell aggregation was entirely similar to that of the undiluted blood.

2. With 1:4 dilution there was a very small amount of cryoglobulin precipitate with approximately proportional diminution in the red blood cell aggregates.

3. With 1:6, 1:8, and 1:10 dilutions there was no precipitate and aggregates of red blood cells were no longer evident.

This behavior was in striking contrast to that of the plasma of the patient with cold autoagglutination where the red blood cells were still aggregated with dilutions of a million parts with normal saline.

C. Sedimentation Rate.—The abnormalities which have been mentioned were gross alterations in the suspension stability ordinarily estimated by observation of the sedimentation rate. An effort was made to determine the effect of the abnormal globulin on the sedimentation rate. Wintrobe tubes were used and observations made by the single one-hour reading technique and by multiple readings at five-minute or shorter intervals to determine the rate of fall during the period of constant fall by the Rourke-Ernstene technique.[3] Potassium oxalate (10 mg. to 10 c.c. blood), Heller-Paul mixture (10 mg. to 10 c.c. blood), sodium citrate (1 c.c. 3.8 per cent to 10 c.c. blood), defibrination and heparin were used for anticoagulation. Observations were made in most instances at

both 37° and 25 to 26° C. Exact observations with potassium oxalate were not possible either at room temperature or at 37° C. because of the rapid pseudo-agglutination. With sodium citrate the delay in precipitation permitted study both at room temperature and at 37° C.

1. For control, observations with both oxalate and citrate were made on the blood of normal subjects to determine the effects of temperatures ranging from 26° to 37° C. The results were in agreement with previously reported studies of Plass and Rourke[4] and Ham.[5] Increase in the environmental temperature from 26° to 37° C. accelerated sedimentation by 15 to 20 per cent. Sodium citrate as the anticoagulant was found to retard sedimentation slightly and to decrease the hematocrit by dilution.

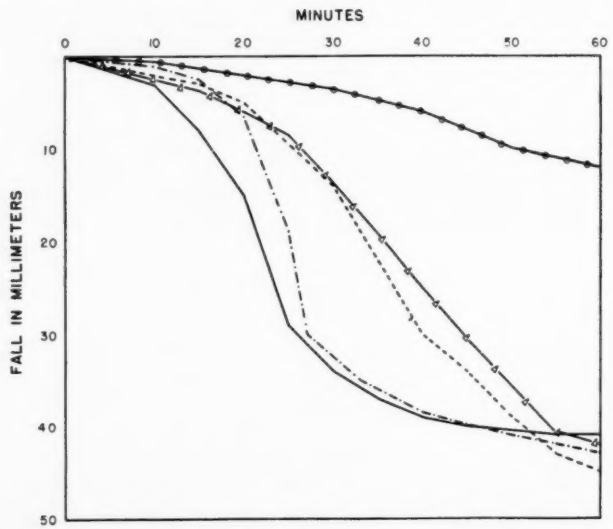

Fig. 2.—Sedimentation rate in cryoglobulinemia. ———, Patient P. I., male, C.S.I. (corrected sedimentation index) = 1.0 mm. per minute. o—o—o, Normal female subject, C.S.I. = 0.3 mm. per minute. △—△—△, Patient B.P., male, C.S.I. = 1.0 mm. per minute. — — —, Patient L.P., female subject, C.S.I. = 1.5 mm. per minute. •-•-•, Patient J.R., male, 2.0 mm. per minute.

2. The sedimentation rate was determined on five patients with cryoglobulinemia, one with Grade I, two with Grade II, according to the classification of Lerner, Barnum, and Watson,[6] and the other two patients with large amounts of cryoprotein. It was found to be markedly increased in all instances. The characteristic feature was a rapid phase of aggregation with a steep curve of precipitous sedimentation during the period of constant fall.

3. More detailed observations were made on the blood of one of the patients with large amounts of cryoprotein as follows.

(a) Very marked acceleration of the corrected sedimentation rate was observed with citrate both at room temperature and at 37° C. and with all anticoagulants at 37° C. The rapidity of fall or sedimentation of the cells was so marked as to present an excellent opportunity for observation of the three phases of sedimentation defined by Fahraeus.[8] *First* there was gross *horizontal aggregation* of cells in masses which in citrated blood was not accompanied by

grossly visible precipitate. In oxalated blood at room temperature prompt "jelling" occurred but usually there was slight settling of 1 to 2 mm. at the top of the column. At 37° C. "jelling" also occurred occasionally, but usually the phase of aggregation could be observed and its association with some precipitate of protein could be demonstrated. In citrated blood following the aggregation phase there was clearly visible descent of the aggregates of red cells with rapid clearing of the supernatant plasma. This corresponds to the *period of constant fall.* The phase of packing was normal in appearance except for the lower part of the layer of cryoglobulin where red blood cells were enmeshed. The cryoglobulin layer could be identified as intermediate in density compared to red and white cells.

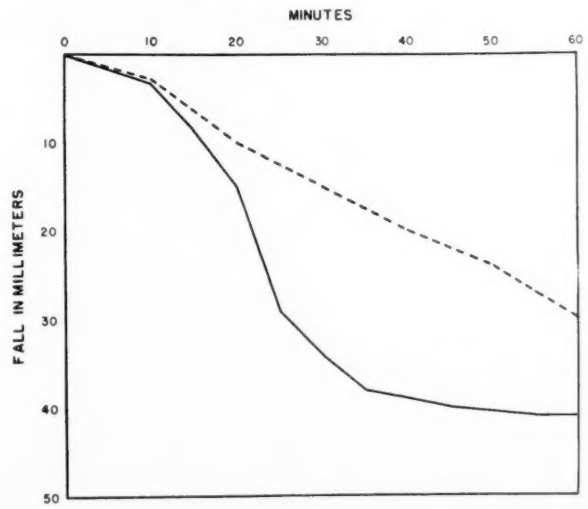

Fig. 3.—Effect of fibrinogen removal on sedimentation rate in cryoglobulinemia. Solid line, citrated blood, Patient P.I., C.S.I.=1.0 mm. per minute. Broken line, defibrinated blood, Patient P.I., C.S.I.=0.15 mm. per minute.

(b) To determine the influence of factors intrinsic in the red cells upon the increased sedimentation rate, the cells were washed twice in saline and resuspended in various liquids. In plasma from a normal person with a homologous blood group (O) the sedimentation rate equaled that of the fall of the normal person's cells in this plasma. Suspension of the washed cells of our patient in normal saline resulted in no fall in one hour. Washed cells from four other patients exhibited no fall in one hour when suspended in normal saline. Suspension of the cells of our patient in 0.85 per cent cryoglobulin solution in saline resulted in a slightly more rapid fall than was observed when a normal person's cells were suspended. The fall, however, was of small magnitude and did not exceed 8 mm. in one hour. The discrepancy may be explained by retained cryoglobulin on the surface of cells of our patient even after they had been washed twice in saline.

It was concluded that the increased sedimentation rate was not due to intrinsic properties of the red blood cells.

(c) To determine the effect of the fibrinogen content of the plasma the sedimentation rate was determined on defibrinated blood. The specimen was collected in a warm syringe and was maintained at 37° C. during defibrination with glass beads and filtration through a gauze square. Sedimentation rates were observed at both 26° and 37° C. The gross amount of cryoglobulin precipitate was unchanged by the defibrination and the sedimentation rate was markedly elevated above the normal values quoted by Ham[7] for defibrinated blood. The fibrinogen content of the plasma on the day of the determination was 0.89 Gm. per cent. This elevation of the plasma fibrinogen level was considered artificial and the result of relatively small amounts of cryoprotein which were precipitated during fibrin removal.

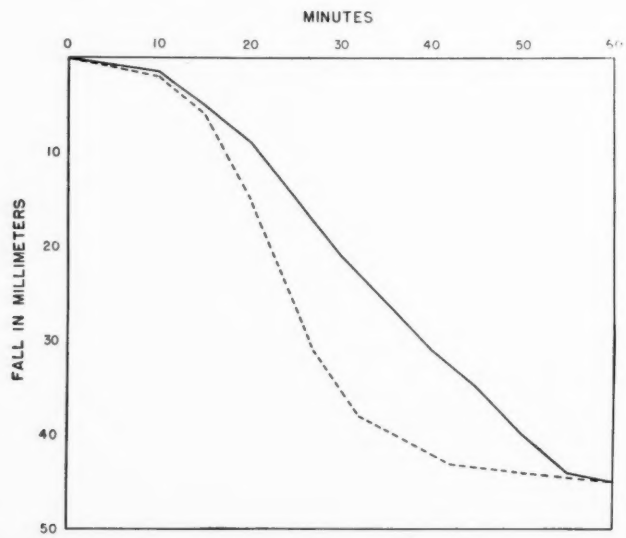

Fig. 4.—Effect of removal of cryoglobulin from plasma on sedimentation rate. Solid line, homologous group cells in plasma supernatant (cryoglobulin-free), C.S.I.=0.6 mm. per minute. Broken line, homologous group cells in unaltered plasma, C.S.I.=1.3 mm. per minute.

It was concluded that the elevated sedimentation rate is due, at least in part, to factors in the plasma other than fibrinogen.

(d) To determine the importance of various plasma factors other than fibrinogen on the sedimentation rate, plasma was obtained from the patient by the usual technique and separated cells were then resuspended in 1:2, 1:4, and 1:8 dilutions of plasma in normal saline.

The rate of fall was compared to that of cells suspended in unaltered plasma. There was a marked acceleration of the corrected rate in the 1:2 dilution of plasma, some retardation in the 1:4 dilution, and marked retardation in the 1:8 dilution. Control of cells suspended in normal saline resulted as before in no sedimentation. With a 1:2 dilution of plasma, the sedimentation was complete in fifteen minutes. This effect, which may be attributable to a decrease in the viscosity, was disproportionate to the effect of similar dilution on the rouleaux-producing factor in the plasma. The influence of marked dilution as

in the 1:8 mixture on rouleaux or aggregate formation is well known.[8] Normal blood with a corrected sedimentation rate of 3.5 mm. per hour did not show a similar acceleration of sedimentation with a 1:2 dilution of the normal plasma and resuspension of the cells. There was marked retardation of fall with all dilutions of the plasma in normal blood.

It was concluded that a factor was present in the plasma which accelerated the sedimentation rate in undiluted blood and which exhibited acceleration initially with low dilutions and retardation with higher dilutions.

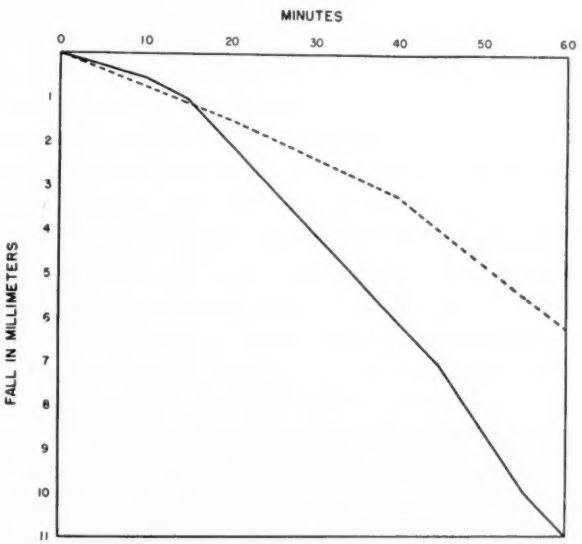

Fig. 5.—Effect of addition of cryoglobulin to normal plasma. Solid line, normal blood as control, C.S.I.=0.15 mm. per minute. Broken line, normal blood with cryoglobulin added to plasma, C.S.I.=0.35 mm. per minute.

(e) To determine the effect on the sedimentation rate of the removal of cryoglobulin from the plasma, washed red cells of the homologous group were resuspended in citrated plasma from our patient. There were two groups of observations. A portion of the cells was suspended in unaltered plasma containing cryoglobulin. Another portion was suspended in plasma from which cryoglobulin had been precipitated by storage at 5° C. overnight. The hematocrits were the same. The corrected sedimentation rate of the blood cells suspended in plasma from which cryoglobulin had been removed was decreased by approximately 100 per cent. The sedimentation in the supernatant cryoglobulin-free plasma was still elevated above normal at a rate of 0.5 mm. per minute which may be compared with the upper limit of normal of 0.3 mm. per minute. This suggested that there remained in the plasma of our patient after cryoglobulin precipitation for twenty-four hours some other factor which accelerated the fall of erythrocytes. From other observations it is now considered probable that complete cryoglobulin precipitation had not occurred at the end of twenty-four hours.

It was concluded that the removal of cryoglobulin from plasma resulted in a significant decrease in the corrected sedimentation rate of resuspended cells.

(f) To determine the effect on the sedimentation rate, cryoglobulin was added to normal blood. Because of its insolubility in normal saline it was necessary to use an aqueous solution of cryoglobulin for these observations. The cryoglobulin used in this experiment was obtained by precipitation at 5°, re-solution in dilute saline and repeated cold precipitation, dialysis, and finally solution in distilled water. Electrophoretic study revealed a mobility resembling a gamma globulin. Unfortunately the specimen used migrated with a slight asymmetry of boundary suggestive of an impurity. This asymmetry was not evident in samples from this same patient studied at a later date.[1] For this reason the data presented in this portion of this report cannot be considered the result of the addition of a homomolecular protein. The addition of 0.2 c.c. of a 1.3 per cent aqueous solution of cryoglobulin to 2 c.c. of normal oxalated blood resulted in an approximately 100 per cent increase over the control in which 0.2 c.c. of distilled water had been added to 2 c.c. of the same blood. The dilution resulted in no visible hemolysis.

It was concluded that the simple addition of cryoglobulin to normal blood profoundly affected the sedimentation rate.

DISCUSSION

It has long been recognized that the suspension stability and sedimentation rate of erythrocytes are affected by the protein content of the suspending fluid. Early work by Fahraeus[9] demonstrated that of the serum proteins albumin exerts relatively little effect, while the presence and concentration of fibrinogen and the globulins are more influential. If the complexity of the blood plasma as a colloidal solution is considered, it would be remarkable if there were not many mechanisms for alteration of the suspension stability of the red cells. All plasma proteins can be considered to have variable effects with varying concentrations. Furthermore, the plasma lipid, the electrolytes, and specifically the hydrogen ion concentration must be of some importance in the control of interfacial tension between the cells and suspending medium. Emphasis has been placed on fibrinogen as the most important factor, the relationships of which were defined by Ham and Curtis[5] and Plass and Rourke.[4] It later became apparent, however, that the sedimentation rate occasionally varied independently of the concentration of fibrinogen as ordinarily determined and that profound acceleration in the fall of erythrocytes might occur with grossly normal fibrinogen values. This raised the question whether the protein fibrinogen itself was the most significant agent or whether some substance of lesser concentration which was precipitated with fibrinogen in the usual methods of analysis might be responsible. The work of Morrison, Edsall, and Miller[10] has indicated the probable complexity of the fibrinogen fraction of plasma. In their subfraction I, they discovered a cold precipitable substance. They suggest that this may be identical with I. R. Morrison's contractinogen,[11] a protein which

was precipitated by cold, redissolved by warming, and was thought by the author to exert a determinant influence on sedimentation rate of erythrocytes. The property of cold precipitability possessed by these proteins might suggest that they are closely related to the cryoglobulin with which we have been concerned. Little is known about the properties of I. R. Morrison's contractinogen, but the protein of Morrison, Edsall, and Miller appeared electrophoretically to be a beta globulin while our protein had properties resembling a gamma globulin. Furthermore, the molecular weight of their protein was much greater than the approximately 190,000 indicated for ours.

Whatever may be the effect of fibrinogen and related substances, it is well known that hyperglobulinemia is usually if not always accompanied by acceleration of the sedimentation rate and by striking abnormalities in suspension stability. Multiple myeloma, the disease in which increase in globulin concentration in the serum is most marked, is so frequently accompanied by rouleaux formation, appearance of "red sand," pseudoagglutination, and increased sedimentation rate that these phenomena have been useful in its clinical recognition. With hyperglobulinemia the factor most obviously affecting the suspension stability of the blood is the aggregation or agglutination of the cells. Although the relation of high globulin content to these abnormalities is very evident, there is no strict proportionality between the two. Furthermore, apparently similar changes in the agglomeration of red blood cells and accelerations of sedimentation rate occur in cases of auto-, cold- and iso-agglutination in which an abnormal globulin is known to be present but in amounts so small as to escape detection by ordinary chemical methods of analysis. Furthermore, the influence may be of such potency that as in the case previously mentioned in this paper the agglutination can still be produced by a serum that has been diluted a million times.

In considering the behavior of the blood from our patient a number of factors must be taken into account. The serum contained a protein which had the remarkable property of spontaneous precipitation and which was present in the extraordinary concentration of 5 to 10 Gm. per cent. The protein was very viscid with a measured relative viscosity of 1.156. Electrophoretic analysis disclosed the presence in the plasma after the cryoglobulin had been removed of two other abnormal proteins with peaks at M and M_1 in amounts respectively of 0.36 to 0.26 and 0.33 to 1.14 Gm. per cent. By the usual method of analysis the fibrinogen content of the plasma was within normal limits on one occasion and elevated on another.

In the behavior of the undiluted blood the spontaneous protein precipitate played a predominant role. It was visible in some specimens even at 37° C. and was massive at room temperatures. At 26° C. it enmeshed and mechanically agglomerated the erythrocyte and presented the appearance of jelling or pseudoagglutination. In some instances this phenomenon contributed to an extremely rapid sedimentation rate while in others the jelled column offered mechanical impedence to the settling of red blood cells.

Precipitation of protein and red blood cell aggregation did not appear to be influenced inordinately by dilution of the blood with an equal volume of normal saline. They were, however, greatly diminished with fourfold dilution and disappeared completely when the blood was diluted six times or more with the salt solution. Removal of the influence of precipitation, however, did not establish normal sedimentation rates.

Throughout the experiments it was evident that the acceleration of sedimentation rate did not depend entirely upon precipitation of protein and agglomeration of cells. It was evident at 37° C. where precipitation was minimal. It was still apparent when blood was diluted beyond the point of visible agglomeration of red blood cells. The residual effect could be attributed in part to the presence of the viscid cryoglobulin in solution, an inference which was supported by the 100 per cent increase in the sedimentation rate when relatively small amounts (0.13 Gm. per cent) of cryoglobulin were added to normal blood. That it may not have been the only factor in the blood of our patient was indicated by the moderate elevation in sedimentation rate which persisted after the removal (twenty-four precipitation) of cryoglobulin from the plasma and which might have been due to the presence of other abnormal proteins (M and M_1) or to abnormalities in the composition of the fibrinogen fraction.

It is not remarkable that a globulin, and particularly one of such great viscidity, should exert an influence upon the sedimentation rate and suspension stability of the red blood cells. It is of particular interest, however, that our cryoglobulin approached chemical and physical homogeneity so closely and that its effect could be studied as a single variable, a rare circumstance since most proteins because of difficulties inherent in methods of separation cannot be isolated in so near the homomolecular state. The spontaneous precipitation of the cryoproteins offers unusual opportunity for the separation and isolation of proteins. Extremely preliminary observations recorded in this paper indicate that other cryoproteins in much smaller concentration may exert a qualitatively similar action on the sedimentation rate and suggest the desirability of more extensive studies on this group of substances.

Before concluding, a note on the method of estimation of the sedimentation rate may be warranted. If one examines the three sedimentation curves in Fig. 3, it is apparent that performance of the test on citrated blood at room temperature with only a one-hour reading would indicate a corrected sedimentation rate by Wintrobe technique of 10 to 11 mm. per hour because of the correction for hematocrit. The obviously bizarre sedimentation of this blood made this form of recording grossly erroneous. Inspection of the curve of fall by the Rourke-Ernstene technique reveals a markedly elevated rate during the period of constant fall which has been regarded as of most importance. The error in the Wintrobe timing method has been pointed out previously by Ham[5] and is attributable to the inclusion of a period of packing in the one-hour observation. It can introduce significant error in the study of blood with low hematocrit

reading. It suggests that if a single reading technique is used, normal blood sedimentation rates should be determined for a thirty-minute single observation or for periods of both thirty minutes and one hour and these intervals used.

SUMMARY

Marked disturbances in suspension stability and sedimentation rate of erythrocytes have been demonstrated in the blood of a patient with multiple myeloma and cryoglobulinemia. At room temperature and below, these are in part attributable to the spontaneous precipitation of the cryoglobulin and the mechanical aggregation of cells, but at 37° C. they are influenced by the presence of the viscid abnormal protein in solution. The study has presented the unusual opportunity of examining as a single variable the effect of an abnormal protein which approached the homomolecular state on the suspension stability and sedimentation of red blood cells.

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ELECTROPHORETIC CHARACTERIZATION OF SERUM FROM RHEUMATOID ARTHRITIS PATIENTS

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ELECTROPHORETIC analyses of the serum proteins in rheumatoid arthritis[1-4] have shown a fairly consistent increase in the proportion of α- and γ-globulins with a relative decrease in the albumin fraction. Dole and Rothbard[2] making serial observations on a single patient and Paul and Routh[4] studying a number of patients found that these abnormalities decreased with clinical improvement. In addition to the changes previously reported, Wallis[3] observed the presence of a T or γ_1 component in the sera of three out of four individuals with rheumatoid arthritis of slight to moderate activity. This component has been observed in horse antitoxic serum[5] and probably also in normal human plasma.[6]

The serum in many cases of rheumatoid arthritis has also been shown to be abnormal serologically in that it markedly enhances the specific agglutination of sheep erythrocytes[7-13] and the erythrocytes of some other species.[14] Waaler[7] found that the factor causing the agglutination of sensitized sheep cells was present in the globulin fraction. It was further shown by Rose and co-workers[8] that the factor could be demonstrated in the electrophoretically separated β-γ-globulin fraction.

In view of these electrophoretic and serologic peculiarities that have been found in rheumatoid arthritis serum, further observations seemed indicated in order to compare electrophoretic patterns with the capacity of serum samples to agglutinate sensitized sheep cells.

METHODS

Electrophoretic analyses were made on twenty samples of serum from a randomly selected group of sixteen patients with rheumatoid arthritis including one case of rheumatoid spondylitis and one case of Still's disease. A conventional Veronal buffer (pH = 8.6)[15] was used and the analysis was made with a Perkin-Elmer Model No. 38 Tiselius electrophoresis apparatus. Samples were diluted 1:4 and run for eighty-five minutes at 5 milliamperes.

RESULTS

A survey of the patterns showed one with a very peculiar protein distribution which could not be correlated with any of the others or any known clinical abnormality. The patterns of three others showed no atypical characteristic.

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One of these was the serum from the patient with Still's disease and another was from the patient with rheumatoid spondylitis. In the remaining twelve, however, there appeared an abnormality in the descending boundary pattern which has no counterpart, as far as we have been able to determine, in any of the analyses we have made by the same method on more than eighty serum specimens from a variety of conditions. Nothing significant could be seen in the ascending boundary. Fig. 1 shows the descending boundary patterns of four of these sera, with a peak lying at the position of the β-globulin similar to that of the "β-anomaly."[16] Table I summarizes certain clinical and laboratory data on all patients in relation to the presence or absence of the abnormal peak. No correlation between size of the peak and titer for sensitized sheep cells or sedimentation rate could be demonstrated. Nor was there any apparent relationship between the peak size and severity of the disease.

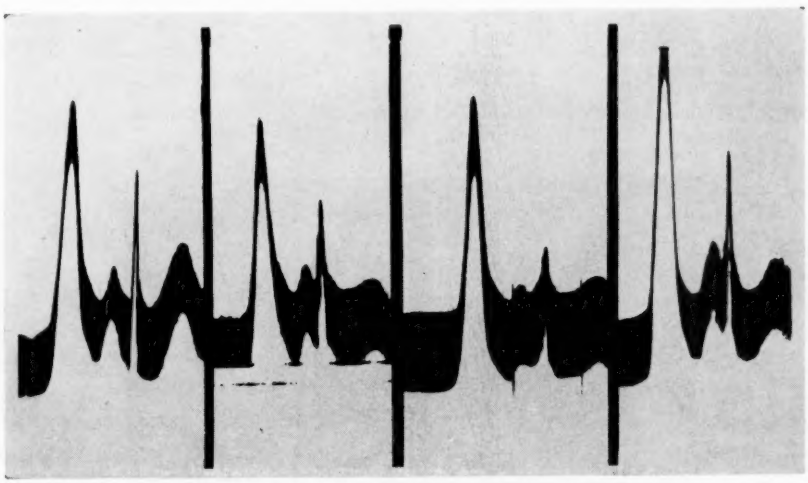

Fig. 1.—Descending electrophoretic patterns of the serum of four patients with rheumatoid arthritis showing the abnormal peak at the position of the β-globulin. Veronal buffer, pH 8.6. From left to right, Patients M. B., W. C. S., J. H., and G. R.

The inference which is drawn from the appearance of the abnormal peak is that it is caused by the interaction of parts of two of the globulin fractions, or parts of one of the globulin fractions and the albumin fraction, in the buffer solution, since no corresponding peak is found in the ascending boundary pattern. A somewhat similar situation is found from time to time with other sera in the so-called "β-globulin disturbance" or "β-anomaly"[16] mentioned. Fig. 2 illustrates the β-anomaly in a typical pattern of a descending boundary. No corresponding abnormality is evident in the ascending boundary. One explanation advanced to account for this disturbance is that a combination of two (or more) serum components occurs under the existing buffer conditions. This produces an extremely dense layer followed by a layer of sparsity, causing a deflection of the optical boundary both above and below the reference position. In the rheumatoid arthritis sera, however, the deflection is predominantly above

TABLE I. SUMMARY OF CLINICAL AND LABORATORY DATA ON RHEUMATOID ARTHRITIS PATIENTS WHOSE SERA WERE EXAMINED ELECTROPHORETICALLY

	PATIENT	AGE (YR.)	SEX	TITER FOR SENSITIZED SHEEP CELLS	E. S. R.‡	DURATION OF DISEASE	SEVERITY OF DISEASE
Patterns showed abnormal peak at position of β-globulin	G. R.	56	F	20	30	5 yr.	Mild
	M. B.	62	F	80	90	1 yr.	Moderate
	W. M.	52	M	1,280	32	7 yr.	Moderate
	J. H.	16	F	640	32	3 yr.	Moderate
	W. C. S.	56	M	640	48	6 yr.	Marked
	B. M.	68	F	160	42	4 mo.	Moderate
	M. E. C.	73	F	10	22	10 yr.	Moderate
	J. H. H.	62	F	20	24	7 yr.	Mild
	L. L. D.	42	F	20	21	8 mo.	Moderate
	W. W. A.	50	F	80	17	6 mo.	Mild
	J. R. G.	55	M	80	46	5 yr.	Moderate
	E. A.	26	M	10	3	2 yr.	Mild
Did not show the abnormal peak	W. T. W.	59	M	20	100	1 yr.	Marked
	C. T.*	43	M	40	65	12 yr.	Moderate
	W. H. J.	70	M	640	117	2 yr.	Marked
	R. G.†	12	M	40	87	8 yr.	Marked

*Spondylitis.
†Still's disease.
‡Erythrocyte sedimentation rate in millimeters per hour (Westergren).

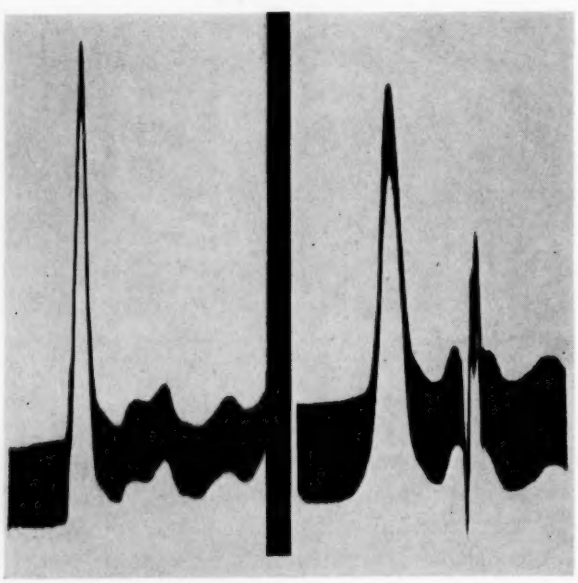

Fig. 2.—Descending electrophoretic patterns of normal serum (left) and serum showing the "β-anomaly" (right). Veronal buffer, pH 8.6.

the reference position, indicating no region of sparsity and a qualitative difference in serum reaction under the measurement conditions. This absence of a region of sparsity could also be occasioned by an overbearing β-globulin concentration which, however, was not present in the rheumatoid arthritis sera examined.

Since the β-anomaly is not as likely to be observed when a phosphate buffer is used, analyses were made using phosphate buffer (pH = 7.4) on a rheumatoid serum which had shown the abnormality when tested with the Veronal buffer. The abnormal peak did not appear with phosphate buffer.

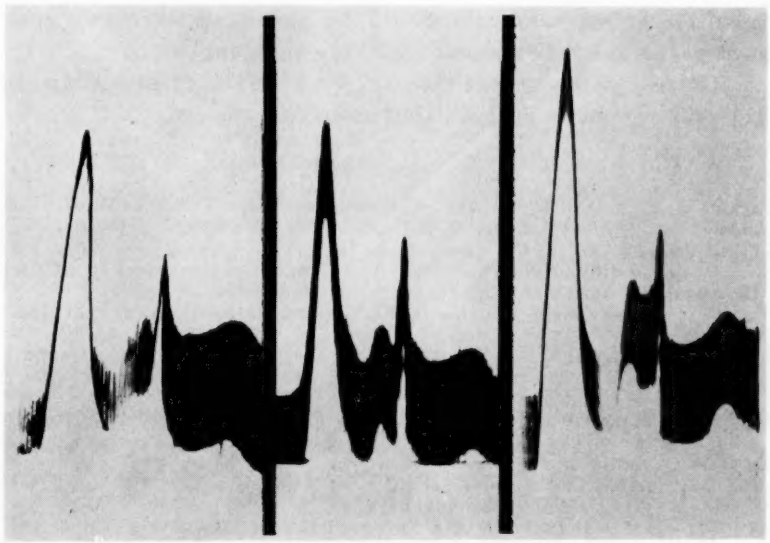

Fig. 3.—Descending electrophoretic patterns of three specimens of serum from Patient W. C. S. obtained (left to right) in April, 1949, June, 1949, and January, 1950. Veronal buffer, pH 8.6.

Electrophoretic patterns were taken on three serum specimens obtained from one patient over a period of nine months. During this time there was only slight improvement in clinical symptoms with gold therapy. Fig. 3 shows the patterns of the descending boundaries; it is noted that the abnormality persists.

DISCUSSION

The failure of Paul and Routh[4] or others to identify the abnormality observed in these cases of rheumatoid arthritis may have been due to their failure to differentiate it from a β-globulin component or from the conventional β-anomaly. In the use of certain modifications of the Tiselius apparatus employed by many investigators the differentiation between a β-anomaly and these peaks might be missed readily.

SUMMARY

Electrophoretic examination of sera from rheumatoid arthritis patients showed a characteristic identifiable peak in the descending boundary pattern in twelve of sixteen cases studied. A similar abnormality has not been observed in numerous electrophoretic analyses of sera from a variety of other disease conditions. This peak, which is interpreted as being caused by a reaction of certain of the serum components in the presence of a barbiturate buffer at pH 8.6, persisted almost unchanged for a period of nine months in one patient

studied in which the clinical symptoms remained substantially the same. No explanation for the absence of this abnormal feature in sera from the two patients with rheumatoid arthritis is advanced. Further studies are indicated to establish whether patients with rheumatoid spondylitis and Still's disease fall within this category since serum from two such patients failed to show this abnormality. The frequency with which this abnormality occurs suggests that electrophoresis might be of some value as a diagnostic aid.

No correlation of the area of the peak with the ability to agglutinate sensitized sheep cells or severity of the clinical symptoms was noted.

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THE TREATMENT OF DICUMAROL-INDUCED HYPOPROTHROMBINEMIA IN MAN WITH EMULSIFIED VITAMIN K_1 ADMINISTERED INTRAVENOUSLY

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THE treatment of Dicumarol-induced hypoprothrombinemia in dogs with an intravenously administered emulsion of vitamin K_1 (2-methyl-3-phytyl-1, 4-naphthoquinone) already has been described.[1] This paper reports studies in the treatment of Dicumarol-induced hypoprothrombinemia in man with similar emulsions of vitamin K_1.

METHOD

The emulsions used contained 50 mg. of vitamin K_1 per milliliter. One emulsion was prepared in this laboratory as described in a preceding paper.[1] The other emulsion was prepared elsewhere.* Both emulsions gave similar results. The prothrombin times† of citrated blood were determined by the Link-Shapiro modification of the Quick method, using human thromboplastin.[1] The emulsion was routinely injected intravenously through a No. 25 needle at a rate of administration which averaged 1 ml. per minute.

Seven patients suffering from a variety of cardiac and vascular ailments who were being treated with Dicumarol in amounts adequate to increase their prothrombin times to levels ranging from 43 to 85 seconds were given the vitamin K_1 emulsion intravenously in doses of from 5 to 20 mg. per kilogram body weight or from 360 to 1,290 mg. per patient. Five patients received the emulsion in one injection and two patients in two equal injections as shown in Table I. Prothrombin times were determined after injection of the emulsion at the intervals indicated in Table I. Dicumarol was discontinued after administration of the emulsion in all cases.

RESULTS

The essential data on each patient are recorded in Table I. A representative response to vitamin K_1 emulsion is shown graphically in Fig. 1.

All patients with Dicumarol-induced hypoprothrombinemia showed a prompt decline in prothrombin time following administration of the vitamin K_1 emulsion. When a dose in the range of 10 to 20 mg. per kilogram was administered, the prothrombin times of all the patients fell to 36 seconds or below in 120 minutes and to 30 seconds or below in five hours. Four of the six patients from whom fifteen-minute specimens were obtained showed a transient, usually slight, prolongation of the prothrombin time similar to the rise in prothrombin time observed in dogs.[1]

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†The prothrombin time in seconds may be translated into per cent of normal activity by reference to Fig. 1 where both scales are given on the ordinate.

TABLE I. THE EFFECT OF AN EMULSION OF VITAMIN K_1 GIVEN INTRAVENOUSLY ON THE PROTHROMBIN TIMES OF SEVEN PATIENTS WITH DICUMAROL-INDUCED HYPOPROTHROMBINEMIA

	VITAMIN K_1		PROTHROMBIN TIME IN SECONDS							
PATIENT	MG. PER KG.	MG. TOTAL	INITIAL	15 MIN.	45 MIN.	60 MIN.	90 MIN.	2 HR.	5 HR.	20 HR.
M. M.	10	438	78		47	37	30	31	24	19
W. M.	15	900	67	74		44	36	36	30	20
J. D. (a)	20*	1,000*	85	101	67		33	28*	20	21
A. W.	5	360	66	65		45	42	36	26	25
L. G.	20†	1,290†	60	66	45	45	36†	34	29	25
J. D. (b)	10	800	43	33		31		31	27	25
J. B.	10	650	51	57		40		31	28	24
Control‡	0	0	60						62	59

*Divided dose—500 mg. initial, 500 mg. at two hours.

†Divided dose—645 mg. initial, 645 mg. at one and one-half hours.

‡Last dose of Dicumarol given twenty-four hours before initial prothrombin determination.

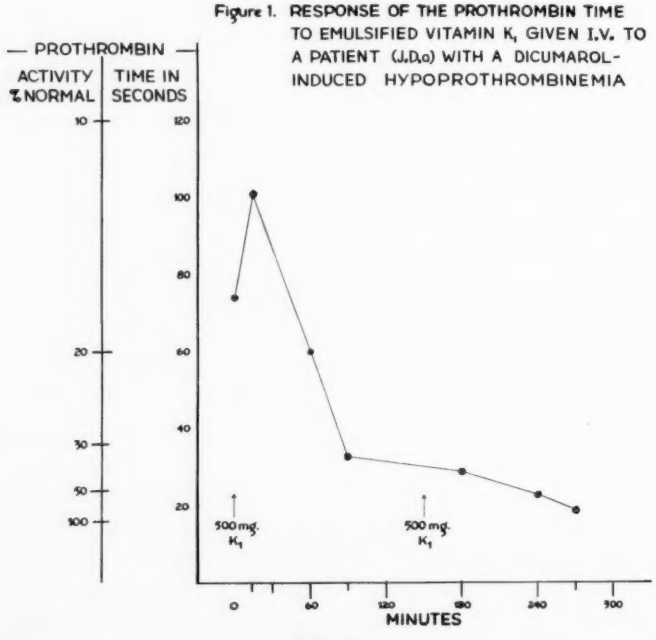

Figure 1. RESPONSE OF THE PROTHROMBIN TIME TO EMULSIFIED VITAMIN K_1 GIVEN I.V. TO A PATIENT (J.D.a) WITH A DICUMAROL-INDUCED HYPOPROTHROMBINEMIA

Fig. 1.

One patient with a prothrombin time of 66 seconds received 5 mg. of vitamin K_1 per kilogram. His prothrombin time subsequently fell to 42 seconds in ninety minutes, 36 seconds in two hours, and 26 seconds in five hours. No rise in prothrombin time was noted at fifteen minutes in this instance.

Of the seven patients who received the vitamin K_1 emulsion intravenously, only one showed an untoward response. This was in an elderly patient (W. M.) who displayed flushing of the face and neck accompanied by a transient rise in blood pressure. The reaction occurred after the administration of 0.8 ml. of the emulsion and rapidly disappeared. Subsequently 17.2 ml. more of the preparation were given without further difficulty.

Unlike the prothrombin times in dogs, which fell promptly to control levels within three hours, the prothrombin times in these patients displayed only a partial recovery in the same interval. After an initial rapid fall to the 28 to 36 second range within 120 minutes, the prothrombin times declined more slowly to a range of 20 to 29 seconds at the five-hour mark. Thereafter the return to levels found in normal individuals was gradual.

In two subjects a second injection of vitamin K_1 emulsion, given after the rapid initial drop in prothrombin time, failed to accelerate appreciably the rate of return to normal values.

DISCUSSION

These studies indicate that Dicumarol-induced hypoprothrombinemia can be altered toward normal more quickly and conveniently in man by the intravenous administration of 5 per cent vitamin K_1 emulsion than by methods previously reported in which similar or greater quantities of vitamin K_1 in a highly dilute suspension were used.[2] Doses of vitamin K_1 ranging from 5 to 20 mg. per kilogram of body weight lowered the prothrombin time from prolonged values to values which fell within the 28 to 36 second range in approximately 120 minutes.

As previously pointed out,[1] emulsions of vitamin K_1 offer distinct advantages over other techniques for administering this material. The action of the preparation is more rapid, and it can be given conveniently by syringe in accurately measured quantities without tedious preparation. At this time little is known about the stability of these emulsions except that emulsions of vitamin K_1 prepared in this laboratory and kept at room temperature out of the light are stable and usable after four months.

The cause of the initial rise in prothrombin time observed in some instances is not known. The species difference between dog and man as evidenced by man's failure to display the same rapid return to a normal prothrombin time as was reported for the dog[1] also is unexplained. It is possible that in man, factors involved in prothrombin production other than vitamin K are altered by Dicumarol therapy, and that these remain uninfluenced by vitamin K_1 administration.

SUMMARY

A 5 per cent emulsion of vitamin K_1 for intravenous administration was successful in reversing Dicumarol-induced hypoprothrombinemia in seven patients and converting prothrombin times of 43 to 85 seconds to times of 28 to 36 seconds in 120 minutes and 20 to 30 seconds in five hours. This preparation acts more rapidly and is more convenient to use than the dilute suspensions previously recommended.

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THE EFFECT OF BENEMID ON PLASMA PENICILLIN LEVELS

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SINCE the advent of widespread penicillin therapy, it has become evident that some method of enhancing the concentration of penicillin in the blood would represent a therapeutic advantage. Because it is well known that about 80 per cent of excreted penicillin is secreted through the renal tubules, it would be desirable to find some safe method to interfere with this tubular mechanism. Diodrast[1, 2] and para-aminohippuric acid[3, 4] when administered by continuous intravenous drip block tubular secretion of penicillin. It is impractical, however, to use drugs requiring intravenous administration when sustained effects are needed. Benzoic acid[5, 6] and carinamide[7-11] when administered orally also are effective but have certain disadvantages which will be discussed later.

Recently, a new compound *p*-(di,n-propylsulfamyl) benzoic acid with the proprietary name of Benemid* has been released for clinical trial. The pharmacologic properties of this compound have been extensively investigated by Beyer and associates.[12] They have demonstrated that the compound is rapidly absorbed from the bowel and that therapeutic doses in experimental animals produce neither systemic nor renal toxicity. In experimental animals, this compound is effective in enhancing the plasma levels of penicillin, P.A.H., phenol red, and certain other related compounds. Recently Boger, Gallagher, and Pitts[13] demonstrated a consistent elevation in plasma penicillin levels in human beings on doses of Benemid averaging about 2 Gm. a day.

Because of the apparent advantage of this drug over carinamide and the obvious therapeutic value of some substance which would enhance penicillin concentrations, further clinical trial seemed warranted. The present study, therefore, is designed to demonstrate the effect of Benemid on plasma penicillin concentrations in the human subject as well as to consider possible toxic effects of this drug during prolonged administration.

PLAN OF INVESTIGATION

Fifteen subjects selected from ward admissions to the Salt Lake County General Hospital were studied. All subjects were below the age of 45 and none showed evidence of renal disease either by history or by examination. All patients received aqueous crystalline penicillin G intramuscularly at eight-hour intervals. In most cases the dose was 200,000 units per injection. Because one occasionally encounters some increase in the plasma penicillin levels after the first twenty-four hours of penicillin administration, control levels were never determined on the first day of penicillin therapy. Benemid was administered orally in varying doses and at varying time intervals. During both the control and the experimental periods, plasma penicillin levels were determined at one, three, five, and seven hours after administration of penicillin.

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RESULTS

Four patients received a single dose of 1 Gm. and one patient received a single dose of 1.5 Gm. of Benemid, one hour before penicillin administration. The results are summarized in Table I and Fig. 1. All but one patient showed some elevation in plasma penicillin levels as compared with those found before

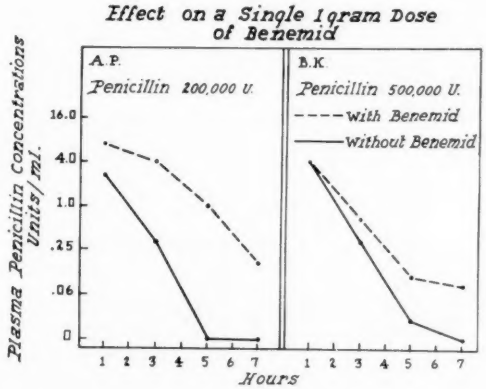

Fig. 1.—The effect of a single dose of Benemid administered orally one hour before penicillin injection. Hours indicate time after the penicillin injection.

Benemid was given, although in general this increase was not of any great magnitude. The effect was most pronounced in the levels taken at five and seven hours. This suggests that Benemid is rapidly absorbed and that some degree of tubular inhibition is achieved promptly. No patient suffered any discomfort from this dose even though it was administered before breakfast.

TABLE I. EFFECT OF A SINGLE DOSE OF BENEMID ON PLASMA PENICILLIN LEVELS

SUBJECT	AGE (YR.)	BENEMID DOSE (GM.)	PENICILLIN DOSE	CONTROL LEVELS				LEVELS FOLLOWING BENEMID			
				1 HR.	3 HR.	5 HR.	7 HR.	1 HR.	3 HR.	5 HR.	7 HR.
B. K.	24	1.0	100,000	0.37	0.06	0	0	0.75	0.09	0	0
A. P.	19	1.0	200,000	3.0	0.37	0	0	8.0	4.0	1.0	0.18
B. K.	24	1.5	500,000	4.0	0.37	0.04	0	4.0	0.75	0.12	0.09
A. P.	21	1.0	200,000	0.5	0.37	0.12	0	1.0	0.5	0.12	0.06
M. P.	22	1.0	200,000	1.0	0.37	0.25	0.04	1.0	0.75	0.25	0.12

Seven patients received multiple doses of Benemid in 0.5 Gm. amounts every six hours. In six cases, three doses of Benemid were given before penicillin levels were determined. In one case, however, only two doses were given. The results are summarized in Table II and Fig. 2. It will be noted that in all cases except one there was an appreciable elevation of the plasma penicillin level which by our method of assay was quite significant. As would be expected there was fairly marked individual variation between patients. It is to be noted again that frequently the enhancement was more marked at the three-, five-, and seven-hour period than at the one-hour period.

TABLE II. EFFECT OF BENEMID ON THE PLASMA PENICILLIN LEVELS FOLLOWING DOSES OF 0.5 GM. EVERY SIX HOURS

SUBJECT	AGE (YR.)	PENICILLIN DOSE	CONTROL LEVELS				LEVELS FOLLOWING BENEMID							
							24 HR.				48 HR.			
			1 HR.	3 HR.	5 HR.	7 HR.	1 HR.	3 HR.	5 HR.	7 HR.	1 HR.	3 HR.	5 HR.	7 HR.
B. M.	23	200,000	2.0	0.75	0.18	0.09	2.0	0.75	0.12	0				
G. H.	44	1,000,000	5.5	3.0	1.25	0.27	32.0	6.0	1.0	0.12				
C. S.	17	200,000	1.0	0.25	0.12	0.04	3.0	1.5	0.37	0.12				
C. A.	44	200,000	1.5	0.25	0.12	0.09	6.0	2.0	0.75	0.25				
A. A.	23	200,000	4.0	0.75	0.12	0.04	4.0	3.0	0.37	0.06				
G. H.	36	200,000	3.0	0.18	0	0	2.0	0.75	0.25	0.18				
M. G.	24	200,000	1.5	0.25	0	0	4.0	1.5	0.25	0.06				
R. C.	22	200,000	2.0	0.5	0.06	0	6.0	1.5	0.37	0.12	6.0	1.5	0.37	0.12
H. T.	37	200,000	2.0	0.5	0.06	0	3.0	0.5	0.09	0	6.0	1.5	0.75	0.18
G. M.	17	200,000	1.0	0.5	0.06	0	2.0	1.0	0.25	0.09	4.0	2.0	0.37	0.09
C. H.	24	200,000	0.75	0.37	0.25	0.09	2.0	1.0	0.37	0.09	4.0	1.0	0.25	0.04
W. W.	45	200,000	1.0	0.37	0.04	0	4.0	1.0	0.18	0.04	6.0	4.0	2.0	1.0

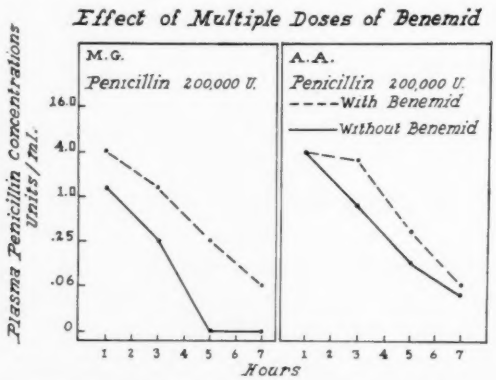

Fig. 2.—The effect of Benemid given at six-hour intervals on the plasma penicillin concentration. In this schedule, the most recent dose of Benemid was given two hours before the test period began. Penicillin was administered at eight-hour intervals.

In order to decide whether the administration of 0.5 Gm. of Benemid every six hours resulted in prompt maximal enhancement of the plasma penicillin levels or if there was a cumulative effect, five patients were treated with this amount of Benemid and plasma penicillin levels were determined on each of two consecutive days. These results are also summarized in Table II and in Fig. 3. It can be seen that most subjects showed higher plasma penicillin levels on the second day than on the first, suggesting that Benemid at this dosage level does not produce maximal tubular inhibition immediately and does have an accumulative effect. This would be in good agreement with Beyer's observation that Benemid is slowly excreted.

One patient who was being treated for subacute bacterial endocarditis caused by an alpha hemolytic streptococcus that was only partially sensitive to 5 units of penicillin per milliliter of plasma, received 2 Gm. of Benemid a day for thirty-five days, or a total of 70 grams. Crystalline penicillin was administered in doses of 2,000,000 units every eight hours. As illustrated in Fig. 4, the plasma penicillin levels were better sustained when Benemid was used

than before its administration. At no time during the administration of Benemid did the patient demonstrate any evidence of toxicity and the urine remained entirely negative throughout this whole period. This would seem to be further evidence that Benemid lacks toxicity in doses of 2 Gm. per diem.

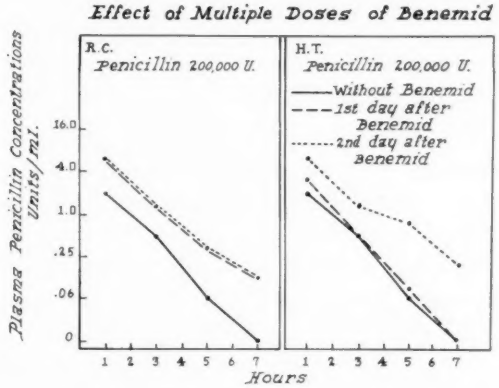

Fig. 3.—The effect of Benemid given every six hours for forty-eight hours. Timing was the same as that in Fig. 2.

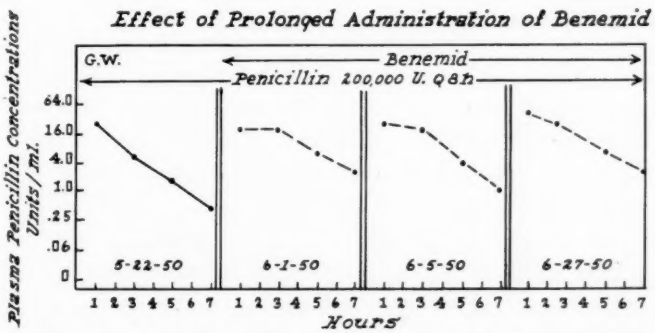

Fig. 4.—The effect of Benemid in doses of 2 Gm. per day over a prolonged period. Note that the enhancement of the plasma penicillin concentration was maintained without alteration of the Benemid dose.

DISCUSSION

From the foregoing data is seems evident that Benemid is an effective agent in delaying the excretion of crystalline penicillin G and that its use is not marred by the undesirable aspects of the agents that have been used previously.

Benzoic acid produces an effect only when there is strict limitation of salt and water intake,[5] a situation that is ordinarily undesirable in the treatment of infections. In addition benzoic acid is not well tolerated by the large majority of patients and hence cannot be used over a prolonged period.[10, 11]

Carinamide is effective without such limitations in salt and water intake and is relatively free from uncomfortable side reactions but has a rather important limitation in that a large number of tablets must be administered at

frequent intervals. In addition, carinamide has one other possible disadvantage when administered over a prolonged period. This compound, administered as the free acid, may be excreted either as the monovalent or bivalent sodium salt. Since the usual dose of carinamide administered to an adult is 24 to 32 Gm. per diem and its molecular weight is 291, it can be calculated that 6 to 8 Gm. of sodium expressed as sodium chloride could be lost each day even if only the monovalent sodium salt were excreted. This could result in severe salt depletion in a patient with a restricted salt intake. We have observed this phenomenon in one patient with subacute bacterial endocarditis.

On the other hand, an adequate enhancement of penicillin levels is achieved even when Benemid is administered in small doses. This drug did not produce discomfort in any of the patients who received it even over a long period of time and certainly gave no evidence of renal toxicity. Its effect seems quite independent of fluid and salt intake, and the required number of pills is sufficiently small to cause the patient no difficulty. Benemid is rapidly absorbed and slowly excreted so that it need be given only at relatively infrequent intervals. As demonstrated in Fig. 3, its full effect with repeated 0.5 Gm. doses is often achieved on the second day rather than on the first, but this also is seen frequently when carinamide is administered and represents no particular therapeutic disadvantage. If immediate alteration of the plasma penicillin level were desired it could be achieved easily by the use of a larger initial dose of Benemid.

The use of Benemid as a therapeutic agent probably will be limited to those circumstances in which high and sustained plasma penicillin levels seem indicated. Its major usefulness would not be that of raising the initial concentration following an injection of penicillin because this can be more easily done by simply giving more penicillin. Its chief value, however, is that of altering the rate of decline of the plasma penicillin level, thus allowing the maintenance of high plasma levels with infrequent administration of penicillin. For these reasons Benemid should represent a useful therapeutic agent in the treatment of certain infections requiring the prolonged administration of large amounts of penicillin. Moreover, it is free of most of the disadvantages of those blocking agents that have been used previously.

SUMMARY AND CONCLUSIONS

p-(di, n-propylsulfamyl) benzoic acid (Benemid), a new benzoic acid derivative, was administered to a group of patients who were receiving penicillin therapy.

Frequent estimation of plasma penicillin levels indicated that higher levels were to be expected during the period of Benemid administration than in control periods.

No toxic reactions to this drug were encountered.

Benemid would seem to be a useful and effective agent for enhancing plasma penicillin levels.

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CLINICAL AND BIOLOGIC SIGNIFICANCE OF PENICILLIN-RESISTANT STAPHYLOCOCCI, INCLUDING OBSERVATIONS WITH STREPTOMYCIN, AUREOMYCIN, CHLORAMPHENICOL, AND TERRAMYCIN

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CLINICAL failures because of the appearance of drug-resistant microorganisms constitute one of the challenging problems of modern chemotherapy. The sulfonamides were quite effective initially in the majority of patients with gonorrhea and in many cases of hemolytic streptococcic disease. The subsequent appearance of resistant strains of the gonococcus and hemolytic streptococcus caused sulfonamide therapy to give way to treatment with penicillin. After several years of clinical evaluation, penicillin resistance has not been a problem with gonorrhea and hemolytic streptococcic disease. But the incidence of penicillin-resistant strains of staphylococcus is increasing at an alarming rate, and many clinical failures with penicillin can be attributed to this biologic phenomenon.

When pencillin first became available for human therapy, there were indications that some strains of staphylococcus were naturally resistant to the drug. At the University of Minnesota Hospitals eight out of sixty-eight cultures or 12 per cent of coagulase-positive strains of staphylococcus obtained from patients prior to the introduction of penicillin were found to be relatively resistant.[1] About the same incidence of penicillin-resistant strains of staphylococcus was being recorded from other clinics.[2, 3] However, it was questioned by clinicians whether penicillin-resistant strains of staphylococcus would constitute a major cause of therapeutic failures with penicillin.[4, 5] But the intensive and widespread use of penicillin, especially in large hospital centers, has been accompanied by the appearance of an increasing number of highly resistant strains of staphylococcus. In support of this statement are the following observations of North and Christie.[6] The in vitro resistance of thirty-one strains of staphylococcus isolated from patients in hospital wards where penicillin was being used was compared with the resistance of 128 strains isolated from the same wards before penicillin was employed. They found that seventeen of the thirty-one strains from the "penicillin wards" were quite resistant to the drug, whereas none of the 128 strains from the "nonpenicillin wards" exhibited the same degree of resistance. Similar observations have been made by others.[7, 8] There is also adequate evidence that patients can be infected initially by penicillin-sensitive strains of staphylococcus, which as a result of

From the Department of Medicine of the University Hospitals and Medical School.
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treatment with penicillin become highly resistant.[9-17] Some of the mechanisms involved in the manifestation of penicillin resistance by staphylococci will be discussed shortly.

It is now well established that this resistance is not of a temporary nature, and resistant organisms are just as invasive as the sensitive parent cocci. This biologic feature of resistance to penicillin which persists from generation to generation without loss of invasiveness is of extreme importance in the epidemiology of staphylococcic infections and can be correlated with the increasing incidence of therapeutic failures with penicillin. A high percentage of healthy individuals may carry in the nasopharynx invasive coagulase-positive staphylococci that are resistant to penicillin, and this reservoir may then serve as a source of infection for other individuals. One of the most significant studies emphasizing the widespread distribution of these penicillin-resistant strains among healthy individuals is that of Martyn[18] in the obstetric department of St. Mary's Hospital in Manchester, England. The nasopharynx and feces of 130 healthy newborn infants were cultured for staphylococci. None of the babies or their mothers had received penicillin. Staphylococci were obtained from the nasopharynx of 62 per cent of the infants, and from the feces of 50 per cent. Of those strains isolated from the nasopharynx, 55.5 per cent were penicillin-resistant, and 58.5 per cent were resistant when cultured from the feces. Beigelman and Rantz[19] carried out a similar study, and found that 30 per cent of the strains of staphylococcus isolated from the nasopharynx of healthy children were penicillin resistant. Martin and Whitehead[20] observed that the incidence of healthy adult carriers of penicillin-resistant strains of coagulase-positive staphylococcus was about 20 per cent.

While it has been emphasized in the literature that penicillin-resistant strains of staphylococcus are most frequently encountered in a hospital population because penicillin is being used in these areas, it should be pointed out that penicillin is rapidly being disseminated far beyond hospital centers. Penicillin is now being administered more frequently in the homes and in rural areas. This means that penicillin-resistant strains will appear more diffusely through the population, the carrier rate of these resistant strains will increase, and the incidence of penicillin-resistant staphylococcic infections will rise. This trend is borne out by many recent observations in widely separated geographical areas, even though the methods of testing for penicillin sensitivity vary from laboratory to laboratory. At the Mayo Clinic, Nichols and Needham[16] observed that 68 per cent of the strains isolated by them from patients were considered to be penicillin-resistant. Beigelman and Rantz[19] in San Francisco found 56.2 per cent of strains tested to be penicillin-resistant, and Finland and associates[17] in Boston have observed an increased incidence in recent years. In England, a few individuals have considered that the number of penicillin-resistant strains has been exaggerated.[21] However, this is contrary to the observations of several other investigators in that country. The most extensive studies have been carried out in London by Barber and associates.[14, 15, 22, 23] Barber and Rozwadowska Dowzenko[15] in summarizing the increased incidence of penicillin-re-

sistant strains state that 14.1 per cent were found to be resistant in 1946, 38 per cent in 1947, and 59 per cent in 1948. An increase in the incidence of penicillin-resistant staphylococci has been reported also from Melbourne, Australia,[6] from Paris,[24] and from Vienna.[7]

The present study was initiated because an increasing number of cases of staphylococcic sepsis were not being benefited by penicillin therapy at the University of Minnesota Hospitals. It was decided to ascertain the incidence of penicillin-resistant strains of coagulase-positive staphylococci by carrying out in vitro tests with penicillin against recently isolated cultures. Because other antibiotics are being employed along with or instead of penicillin, simultaneous in vitro tests were done with penicillin, streptomycin, chloramphenicol (Chloromycetin), aureomycin, and terramycin. These comparative findings are being reported along with their therapeutic implications. In addition, some of the more pertinent clinical aspects of the mechanism, or mechanisms, by which staphylococci overcome the lethal action of penicillin will be summarized briefly.

MATERIALS AND METHODS OF STUDY

The 104 strains of coagulase-positive staphylococci studied were first isolated in the Bacteriology Laboratory of the University Hospitals. Stock preparations of the potassium salt of crystalline penicillin G, streptomycin hydrochloride, chloramphenicol (Chloromycetin), and terramycin hydrochloride were each prepared with sterile physiologic saline solution and stored in a refrigerator. It was necessary to prepare fresh solutions of aureomycin daily in a concentration of 10,000 μg per milliliter. Solutions of penicillin and streptomycin were made up every two weeks in a concentration of 50,000 units per milliliter and 50,000 μg per milliliter respectively. Both chloramphenicol and terramycin displayed more stability and stock solutions were prepared monthly in concentrations of 250 μg per milliliter.

The sensitivity of each strain of staphylococcus for each of the five antibiotics was determined simultaneously by a tube-dilution method. Twofold dilutions of the drugs were employed ranging in a final concentration in each tube from 0.0078 μg per milliliter to 4,000 μg for the antibiotics except for penicillin and for chloramphenicol. Because of the insolubility of chloramphenicol the maximum concentration with this drug was 250 μg per milliliter. The same numerical concentrations were expressed in terms of units for penicillin. An eighteen-hour broth culture of the organism to be tested was diluted 1 to 100 in tryptose phosphate broth and 0.5 ml. added to each of several tubes (13 by 100 mm.) containing twofold serial dilutions of each drug in 0.5 ml. of saline. Repeated colony counts indicated that this inoculum contained 500,000 to 1,000,000 organisms. This is a larger inoculum than that recommended by Luria[25] and by Parker.[26] Results obtained with a smaller inoculum may be very misleading for the clinician, and this will be commented upon later. The mixtures of organisms and antibiotic were incubated at 37° C. for twenty hours, and the end point in each sensitivity test was determined by selecting the lowest concentration of drug that prevented visible growth.

Penicillinase production by the resistant strains of staphylococcus was determined quantitatively by a previously described method.[27]

RESULTS

The results of the in vitro tests with the 104 strains of staphylococci are presented in Tables I and II. For purposes of comparison each of the strains has been numbered.

Penicillin-Resistant Strains.—As seen in Table I, forty-six of the 104 strains, or 44.2 per cent, were inhibited in growth by 0.5 unit per milliliter or less of penicillin. These may be designated as sensitive strains of staphylococci. An outstanding feature of this study is that forty-five of the 104 strains or 43.2 per cent required more than 62.5 units per milliliter of penicillin to inhibit growth. These strains were considered to be highly resistant to the action of penicillin. It is of interest that 87.4 per cent of all the strains were either quite sensitive to penicillin or highly resistant. On the basis of the genetic studies of Demerec[28] it would have been anticipated that a gradual step-wise increase in resistance would be manifested with all degrees of resistance being more or less equally represented, rather than the preponderance of strains being grouped at the extremes of sensitivity or resistance and with only a few scattered between. When the forty-four strains that grew in the presence of 62.5 units per milliliter of penicillin were studied further, as shown in Table II, twenty-five of the strains, or 24 per cent of the total, required 500 or more units per milliliter of penicillin to inhibit growth. Four strains required 2,000 units per milliliter before growth was inhibited, and 4,000 units per milliliter of penicillin were necessary before the growth of the two most resistant strains was inhibited.

The penicillinase produced by the resistant strains inhibited large concentrations of penicillin. Representative results with sensitive strains and highly resistant cultures are presented in Table III and reveal that no penicillinase production was demonstrated for the sensitive cells, whereas the penicillinase from the resistant organisms was so active that penicillin in a concentration of 1,000 units per milliliter had no effect on the growth of a sensitive strain of staphylococcus.

In several instances, a highly resistant strain of staphylococcus was isolated from a patient who had been receiving penicillin without apparent benefit. As noted in Tables I and II a number of strains were not only resistant to penicillin but also to one or more of the other antibiotics. Resistance to other antibiotics was frequently associated with the previous administration of these antibiotics. On the other hand, some penicillin-resistant strains were sensitive to one or more of the other antibiotics. These observations can be illustrated by referring briefly to the clinical status of some of the patients. Strains 40 and 42 possessed the greatest degree of penicillin resistance. Strain 40 was isolated from a furuncle of a 30-year-old man who had had five admissions to the hospital because of leucemia and recurrent infections of the skin. He had received large quantities of penicillin, streptomycin, and dihydrostreptomycin. It is seen in Table II that Strain 40 was not only resistant to penicillin but was also extremely resistant to streptomycin. He had also received aureomycin, but this was not associated with resistance to this antibiotic. The patient improved when given neomycin.[29] Strain 42 was obtained from the blood of a 72-year-old man who had congenital hemolytic anemia and benign prostatic hypertrophy. He had received large amounts of penicillin and aureomycin. Examination of Tables I and II shows that this strain was highly resistant to streptomycin as well as to penicillin, but 15.6 μg per milliliter of aureomycin inhibited growth.

TABLE I. COMPARATIVE IN VITRO RESISTANCE OF 104 STRAINS OF COAGULASE-POSITIVE STAPHYLOCOCCI TO FIVE ANTIBIOTICS

μG OR UNITS PER ML. OF ANTIBIOTIC NECESSARY TO INHIBIT GROWTH	PENICILLIN (UNITS PER ML.)	AUREOMYCIN (μG PER ML.)	CHLORAMPHENICOL (μG PER ML.)	TERRAMYCIN (μG PER ML.)	STREPTOMYCIN (μG PER ML.)
<0.5	1, 2, 6, 10, 11, 12, 13, 14, 18, 20, 21, 23, 24, 25, 26, 28, 32, 38, 44, 45, 46, 48, 49, 53, 54, 58, 59, 60, 61, 62, 63, 67, 69, 74, 76, 79, 80, 84, 86, 87, 89, 90, 92, 93, 95, 104				
0.5		14, 15, 30, 34, 35, 38, 50, 51, 72		22, 92, 96, 97	6
1.0		2, 5, 6, 9, 10, 11, 12, 13, 29, 31, 32, 37, 40, 44, 45, 46, 48, 49, 52, 53, 54, 55, 56, 62, 64, 80, 86, 87, 88, 92, 95, 100		1, 2, 6, 8, 11, 14, 15, 30, 32, 34, 37, 40, 50, 51, 52, 54, 55, 56, 58, 60, 62, 64, 67, 70, 78, 83, 85, 88, 89, 90, 91, 94, 98, 99	23
1.9		1, 3, 7, 8, 19, 20, 21, 23, 26, 28, 33, 36, 39, 43, 47, 57, 60, 67, 70, 73, 74, 75, 76, 78, 85, 103, 104		3, 5, 7, 9, 10, 12, 13, 18, 19, 20, 23, 24, 25, 26, 28, 29, 31, 33, 35, 36, 38, 43, 44, 45, 46, 47, 48, 49, 53, 57, 59, 63, 65, 66, 69, 71, 72, 73, 74, 75, 76, 77, 79, 80, 81, 82, 84, 86, 87, 93, 95, 100, 101, 103	12, 14
3.9	7, 55	16, 18, 22, 24, 25, 41, 58, 59, 61, 63, 65, 66, 68, 69, 71, 77, 79, 81, 82, 83, 84, 89, 93, 94, 96, 97, 98, 99, 101	22	21, 39, 61, 68, 104	10, 13, 26, 58, 67, 83, 93
7.8	29, 47	90, 91	1, 6, 11, 19, 26, 31, 34, 42, 61, 83, 96, 97, 98, 99	16, 41	2, 3, 7, 21, 22, 36, 38, 45, 52, 60, 61, 62, 65, 80, 84, 97, 101, 102, 104

15.6	4, 71	17, 27, 42, 102	2, 3, 4, 5, 7, 10, 12, 13, 14, 15, 18, 20, 23, 27, 29, 30, 32, 33, 35, 36, 37, 40, 41, 44, 45, 46, 47, 48, 49, 50, 51, 52, 54, 55, 57, 58, 60, 62, 63, 64, 65, 67, 70, 71, 72, 74, 75, 76, 78, 81, 82, 84, 85, 86, 87, 89, 101, 102, 103			1, 8, 16, 17, 18, 25, 28, 29, 32, 37, 41, 43, 47, 48, 53, 54, 55, 56, 57, 59, 63, 66, 68, 69, 71, 73, 75, 77, 79, 82, 86, 87, 88, 89, 91, 94, 95, 96, 98
31.2	83, 98		9, 16, 17, 21, 24, 25, 28, 38, 39, 43, 53, 69, 79, 80, 88, 90, 91, 92, 93, 94, 95, 100, 104			4, 9, 11, 20, 24, 39, 46, 49, 74, 76, 90, 92, 100
62.5	16, 22, 35, 65, 73, 97	4	8, 56, 59, 66, 68	27, 42, 102		44, 81
>62.5	3, 5, 8, 9, 15, 17, 19, 27, 30, 31, 33, 34, 36, 37, 39, 40, 41, 42, 43, 50, 51, 52, 56, 57, 64, 66, 68, 70, 72, 75, 77, 78, 81, 82, 85, 88, 91, 94, 96, 99, 100, 101, 102, 103		73, 77	4, 17		5, 15, 19, 27, 30, 31, 33, 34, 35, 40, 42, 50, 51, 64, 70, 72, 78, 85, 99, 103

TABLE II. COMPARATIVE IN VITRO RESISTANCE OF 47 STRAINS OF COAGULASE-POSITIVE STAPHYLOCOCCI TO THREE ANTIBIOTICS. ALL OF THESE STRAINS WERE PREVIOUSLY FOUND TO BE RESISTANT TO MORE THAN 62.5 μG OR UNITS PER MILLILITER OF AT LEAST ONE OF THE ANTIBIOTICS

μG OR UNITS PER ML. OF ANTIBIOTIC NECESSARY TO INHIBIT GROWTH	PENICILLIN (UNITS PER ML.)	TERRAMYCIN (μG PER ML.)	STREPTOMYCIN (μG PER ML.)
125	37, 75, 77, 81, 94, 101, 102, 103	4, 17	103
250	17, 31, 34, 36, 72, 88, 96, 99, 100, 39, 66		35, 72
500	3, 8, 9, 15, 30, 41, 43, 64, 68, 78, 82, 85, 91		
1,000	5, 19, 52, 56, 57, 70		
2,000	27, 33, 50, 51		15, 64, 78, 85
3,000	40, 42		
4,000			31, 51, 70, 99
>4,000			5, 19, 27, 30, 33, 34, 40, 42, 50

This strain was quite resistant to terramycin, though the patient had not received this drug. There was no evidence that he had received streptomycin. The patient recovered from his illness.

Strains 43 and 93 were of particular interest since they were isolated from the blood of each of two patients with acute bacterial endocarditis. The patient from whom Strain 43 was obtained had been receiving massive doses of penicillin without influencing the bacteremia. The in vitro sensitivity test revealed that this penicillinase-producing strain required 500 units per milliliter of penicillin for the inhibition of growth, but only 1.9 μg per milliliter of aureomycin was necessary. The patient was then given aureomycin intravenously,

TABLE III. COMPARATIVE ANTIPENICILLIN ACTION OF 0.05 MG. OF DRIED CELLS ("PENICILLINASE") DERIVED FROM EACH OF SENSITIVE CULTURES OF STAPHYLOCOCCUS AND PENICILLIN-RESISTANT STRAINS. NOTE THAT NO PENICILLINASE WAS DEMONSTRATED FOR SENSITIVE CULTURES

NUMBER OF STRAIN	UNITS PER ML. OF PENICILLIN REQUIRED TO INHIBIT GROWTH OF STRAINS BEING TESTED FOR PENICILLINASE	UNITS PER ML. OF PENICILLIN REQUIRED TO INHIBIT GROWTH OF SENSITIVE STRAIN OF STAPH. IN PRESENCE OF 0.05 MG. OF DRIED CELLS
Sensitive Strains		
1	0.03	0.0016
12	0.06	0.015
13	0.06	0.008
14	0.125	0.03
Resistant Strains		
27	2,000.	>1,000.
33	2,000.	>1,000.
40	3,000.	>1,000.
42	3,000.	>1,000.
50	2,000.	>1,000.

and coincident with this there was an abrupt improvement in her condition and subsequent recovery. Although Strain 93 was quite sensitive to penicillin, the patient from whom the organism was obtained expired because of a brain abscess which developed during treatment with penicillin.

Strain 27 was cultured from the sputum of a 23-year-old man subsequent to therapy with penicillin, streptomycin, and aureomycin. He entered the hospital because of acute meningitis of undetermined etiology from which he recovered. He also had pneumonia, which was associated with a chronic bronchiectasis. It is to be noted in Table II that Strain 27 was highly resistant to penicillin and to streptomycin. Growth of this strain was inhibited by 15.6 μg per milliliter of aureomycin. Although the patient had never received terramycin, a concentration of 62.5 μg per milliliter was necessary for inhibition of growth. This strain was sensitive to chloramphenicol.

The resistance of Strain 5 is another instance of extreme resistance being correlated with antibiotic therapy. This strain was cultured from the urine of a 69-year-old man who had benign prostatic hypertrophy, renal calculi, and pyelonephritis. He had received abundant quantities of penicillin and streptomycin for a long period of time prior to isolation of the staphylococci. Tables I and II show that Strain 5 was highly resistant to penicillin and to streptomycin, but sensitive to aureomycin, chloramphenicol, and terramycin.

Although several instances can be cited in which the evidence indicated that the appearance of penicillin-resistant staphylococci followed therapy with the drug, this was by no means applicable to all of the resistant strains. Further clinical studies have revealed that individuals who have never received penicillin are apparently becoming infected with these resistant strains.

Streptomycin-Resistant Strains.—Preliminary experiments showed that the same in vitro results were obtained whether streptomycin or dihydrostreptomycin was used. Examination of the comparative results in Tables I and II reveals that the growth of only two strains of staphylococci was inhibited by 1 μg per milliliter or less of streptomycin. Contrary to expectations, a more graduated resistance was exhibited by the 104 strains in comparison to the results with penicillin. There were twenty strains or 19.2 per cent that required more than 62.5 μg per milliliter for the inhibition of growth. This is considerably less than the 43.2 per cent of strains that were highly resistant to penicillin. It is of importance that every one of the strains highly resistant to streptomycin also demonstrated extreme resistance to penicillin. Of particular interest are the nine strains that grew in the presence of over 4,000 μg per milliliter of streptomycin (Table II).

It is seen in Table II that every one of the streptomycin-resistant strains that required more than 62.5 μg per milliliter for inhibition of growth was relatively sensitive to chloramphenicol, and without exception all of these strains were sensitive to aureomycin. With the exceptions of Strains 27 and 42, these streptomycin-resistant strains were also quite sensitive to terramycin.

Aureomycin-Resistant Strains.—Examination of Table I demonstrates that with one exception all of the strains of staphylococcus were susceptible to the

action of aureomycin in concentrations that could be approached therapeutically. Strain 4 required 62.5 μg per milliliter of aureomycin before growth was inhibited. Strain 4 was obtained from a child who had had a chronic staphylococcic infection of the liver and skin for many months. This child had received aureomycin for several weeks prior to isolation of this strain of staphylococcus. It might be assumed that resistance to aureomycin developed during this intensive therapy.

Terramycin-Resistant Strains.—While only one strain was considered to be highly resistant to aureomycin, there were five strains highly resistant to terramycin (Tables I and II). Strain 4, which was the one strain resistant to aureomycin, was even more resistant to terramycin. Strain 17 required 15.6 μg per milliliter of aureomycin to inhibit growth, but 125 μg of terramycin were necessary. Though the sensitivities of the strains to terramycin paralleled those obtained with aureomycin, there were the foregoing important exceptions in which highly resistant strains were encountered with terramycin, but not with aureomycin. No strains were found to be resistant to aureomycin but sensitive to the action of terramycin. None of the patients from whom the staphylococci were isolated had received terramycin.

Chloramphenicol-Resistant Strains.—Referring to Table I, it is seen that on a weight for weight basis, all of the strains of staphylococci were more resistant to chloramphenicol than to either aureomycin or terramycin. Because of the insolubility of higher concentrations of chloramphenicol in water, sensitivity tests could not be carried on beyond concentrations of 250 μg per milliliter. Strains 73 and 77, which were resistant to penicillin, were also very resistant to chloramphenicol. In addition, five other strains (8, 56, 59, 66, 68) required 62.5 μg per milliliter of chloramphenicol before growth was inhibited. Four of these five strains were also resistant to penicillin. As far as could be determined, the resistance of these strains to chloramphenicol did not appear to be related to therapy of the patients with the drug prior to isolation of the organisms. It is of interest that Strain 56, which was resistant to both penicillin and to chloramphenicol, was isolated from the pharynx of a baby girl one week after birth. This infant had received no antibiotic therapy. In the interpretation of in vitro tests with chloramphenicol it is to be recalled that corresponding high concentrations can be achieved readily in the blood of patients following oral administration of the drug.

DISCUSSION

The Mechanism of Penicillin Resistance With Particular Reference to the Role of Penicillinase.—This investigation has confirmed the observation of others in that the incidence of penicillin-resistant strains of staphylococcus is on the increase. Furthermore, the magnitude of the resistance of many of the strains poses an alarming problem in therapy. There is no doubt that the appearance of highly resistant strains of staphylococcus is related to the widespread use of the antibiotic. Therefore, before discussing the therapy of staphylococcic infections with antibiotics, it is desirable to present briefly the basic mechanism

or mechanisms involved in the resistance of staphylococci to penicillin. It is particularly essential from the clinical point of view to call attention to the influence of the enzyme penicillinase on the manifestation of penicillin resistance. It is not the purpose of this report to review all of the possible mechanisms involved in penicillin resistance. Such a discussion is intimately tied up with the mode of action of penicillin. Able reviews on the subject include the comprehensive review of Florey and associates[30] and that of Work and Work.[31]

A fundamental concept concerning the appearance of penicillin-resistant strains of staphylococci was offered by Demerec,[28] who applied genetic principles to the study of the problem. He concluded that within a large population of any penicillin-sensitive strain of staphylococcus there are a few naturally occurring and relatively resistant variants. When such a culture is exposed to penicillin, the sensitive organisms are destroyed, but the resistant variants not only remain viable but reproduce in large numbers. Among their progeny new variants occur which exhibit a slightly higher degree of resistance. Thus, in a step-wise manner variants emerge as a result of the selective action of the drug which are resistant to high concentrations of penicillin. This concept has been widely accepted as a valuable tool in the study of penicillin resistance. But this expression of resistance on a classic genetic basis leaves much to be desired in the way of explaining the underlying biochemical reactions responsible for the increased tolerance to penicillin. From a clinical standpoint, the elaboration of penicillinase by resistant strains of staphylococci is of major importance as an added explanation for the therapeutic failure of penicillin. It is to be emphasized that the production of penicillinase is not claimed to be the sole biochemical mechanism responsible for resistance. But many investigators have stressed the fact that all of the resistant strains of staphylococcus isolated from human cases have produced penicillinase.[8, 15, 18, 22, 24] This was also true in the present study.

Abraham and Chain[32] first described penicillinase, which was an enzyme elaborated by *E. coli* that destroyed penicillin. Kirby[33, 34] first demonstrated an inhibitor of penicillin activity obtained from resistant strains of staphylococcus. In a review on staphylococcic penicillinase, Spink and Ferris[27] concluded that the resistance of staphylococci was related to the speed with which penicillinase was produced, to the potency of the penicillinase, and to the time which a given concentration of penicillin took to destroy the bacterial cells. Bondi and Dietz,[35] in a subsequent report on the significance of penicillinase in penicillin resistance, emphasized the speed with which staphylococcic penicillinase was produced rather than the amount of the enzyme. But a more controversial aspect of the subject was raised by Luria,[25] who confirmed an earlier observation that the in vitro resistance of staphylococci to penicillin was quantitatively related to the size of the inoculum used.[1] He found that the individual cells from a penicillinase-producing strain of staphylococcus were sensitive to penicillin, but when a larger inoculum was used, resistance was manifested because of the accumulation of penicillinase from a large number of organisms. It is important to point out that use of a large inoculum from a sensitive strain will

not significantly alter the end point of the sensitivity test. This has been demonstrated in the present investigations, as well as in previous studies.[27] Luria recommended that in vitro tests for determining penicillin sensitivity should take advantage of a small inoculum of organisms, thus excluding the influence of penicillinase. Parker[26] also noted the influence of the size of the inoculum upon the results of in vitro tests, and Gilson and Parker,[36] utilizing quantitative and reproducible methods, demonstrated that with a large inoculum there was a good correlation between penicillin resistance and the quantity of cellular penicillinase, but when only a few hundred cells were used in a test this correlation disappeared. From a practical viewpoint, Gilson and Parker have shown that when the number of staphylococci is large and the amount of penicillin is limited, penicillinase is a very important mechanism in the resistance of a strain.

In view of the tendency for staphylococci to aggregate in large numbers in the tissues and the desirability of having effective concentrations of penicillin for destroying these organisms, the clinician must be supplied with information from an in vitro penicillin-sensitivity test in which due consideration has been given to the optimum activity of penicillinase. In performing such a test with an inoculum of only a few thousand organisms, a false sense of security may be conveyed to the clinician, and the misleading information can very well lead to a therapeutic catastrophe. This has been emphasized by the observations of Jeffery, Levitt, and Topley[37] who reported the failure of penicillin to control osteomyelitis because of the presence of a penicillinase-producing strain. When a million organisms were used in an in vitro test the strain appeared quite resistant, but not when 1,000 cells were employed. The influence of penicillinase as a cause of therapeutic failure was further stressed by Martin and associates,[24] who failed to eradicate a bacteremia in a patient following the daily injection of a billion units of penicillin; but a cure was obtained with streptomycin. A penicillinase-producing strain of staphylococcus was isolated from this patient. Experience at the University Hospitals has emphasized repeatedly the failure of penicillin to control staphylococcic sepsis because of the presence of resistant staphylococci that produce penicillinase.

Not only does the utilization of a large inoculum, such as 500,000 cells, in an in vitro test readily detect resistance due to the production of penicillinase, but such an inoculum also more accurately mirrors the usual character of the infection for which penicillin is administered. Staphylococci tend to localize and provoke abscesses in various tissues of the body, producing a relatively avascular area of suppuration containing tremendous numbers of organisms. Because of the reduced blood supply the amount of penicillin reaching these foci is limited and much of the antibiotic which does reach the lesion may be destroyed by penicillinase. Experience has shown that the presence of penicillinase-producing staphylococci in areas of the lung and bone with a diminished supply of blood will prevent response to tremendous quantities of parenterally administered penicillin. Likewise, staphylococci that elaborate penicillinase

may localize deep in an ulceration of the endocardium and persist for long periods in the presence of the highest blood concentrations of penicillin obtainable.

Treatment of Infections Due to Penicillin-Resistant Staphylococci.—A staphylococcic infection can be managed more intelligently if information is at hand on the in vitro sensitivity of the invading strain to penicillin. There is at least a rough correlation between in vitro sensitivity and what can be anticipated therapeutically with penicillin. In many instances such information is not available, but the clinician fearing that the offending staphylococcus is penicillin resistant will administer large amounts of the drug to a patient. He concludes that if a sensitive strain is present no harm has been done with the larger doses. The possibility has been raised that the indiscriminate administration of large amounts of penicillin may have some therapeutic disadvantages even in those cases where penicillin-sensitive strains of staphylococci are present. Eagle and Musselman[38] have called attention to a paradoxical phenomenon in which excessive concentrations of penicillin are less effective in vitro against some strains of staphylococci than smaller amounts. They observed a narrow zone of drug concentration that yielded optimum results.

While the use of adequate amounts of penicillin may control some cases of staphylococcic sepsis due to moderately resistant organisms, the tendency in clinical practice is either to administer penicillin along with another antibiotic or to use another agent alone. There is no question that staphylococcic infections have yielded to a penicillin-streptomycin combination after therapy with only penicillin had been attended by failure. The problem of administering antibiotics simultaneously in staphylococcic and other infections should be further investigated in view of recent reports. Jawetz and co-workers[39, 40] have shown that chloramphenicol will interfere with the optimum killing effect of penicillin both in vitro and in vivo for certan strains of enterococci, and Lankford and Lacy[41] have demonstrated that various concentrations of penicillin and aureomycin, and streptomycin and aureomycin, will diminish the anti-staphylococcic in vitro action of the individual drugs. This inhibitory effect appeared to be related to the relative concentrations of the drugs employed in the mixtures.

Streptomycin is an effective agent for the treatment of staphylococcic sepsis, but the data in Table I show that a significant number of strains are quite resistant to the antibiotic. In addition, many of the strains highly resistant to penicillin were also quite resistant to streptomycin. Streptomycin has the additional disadvantage that the staphylococci, as well as other organisms, can become highly resistant to its action very rapidly. The rapid appearance of resistant organisms during treatment with streptomycin or dihydrostreptomycin is much more of a therapeutic problem than that associated with penicillin therapy.

Aureomycin has been reported to be quite effective in staphylococcic infections where penicillin has failed to control infections due to the presence of penicillin-resistant organisms.[16, 42-44] As noted in Table I, only one aureomycin-

resistant strain of staphylococcus was encountered in the present study. Recent studies would indicate that the appearance of aureomycin-resistant staphylococci involves a step-wise and gradual development rather than the rapid and abrupt manifestation of resistance that occurs with streptomycin.[41, 45] Since the study on the present series of staphylococcic cultures has been completed, four cases of staphylococcic infection have been encountered at the University Hospital where the evidence pointed to the development of aureomycin-resistant strains as a result of treatment with this drug.

As noted in Table I, on a weight for weight basis staphylococci were more resistant to the action of chloramphenicol than to aureomycin. A few strains of staphylococci that were highly resistant to chloramphenicol have been isolated from patients. Chloramphenicol has not appeared to be as uniformly as effective as aureomycin in the therapy of staphylococcic infections, but further clinical trial with this agent should be carried out.

Terramycin is active against a wide range of microorganisms including staphylococci.[46, 47] While the present study demonstrated a close correlation between the antistaphylococcic action of aureomycin and terramycin as seen in Table I, there were some notable exceptions. Strains 27, 42, 102, and 17 were markedly resistant to terramycin and to penicillin but sensitive to the action of aureomycin. This latter observation may be of significance clinically, since investigations carried out more recently with strains of staphylococcus not included in this report revealed several additional strains highly resistant to terramycin, but relatively sensitive to aureomycin. This manifestation of terramycin resistance has not been associated with terramycin therapy. These preliminary studies suggest that terramycin-resistant staphylococci might offer more of a clinical problem than that which has occurred with aureomycin.

Bacitracin is another antibiotic that has been recommended for the treatment of penicillin-resistant staphylococcic infections.[48, 49] The present study did not include comparative observations with bacitracin because this antibiotic has been administered systemically to only a few patients at the University Hospitals. The drug proved to be nephrotoxic under these circumstances, and it was found that penicillin-resistant staphylococcic infections could be controlled with other less toxic agents. When Pulaski and Connell[50] treated twenty-six patients with bacitracin parenterally, twenty-four of the patients manifested some nephrotoxic phenomena. Bacitracin is devoid of this toxic reaction when applied directly to superficial lesions. Under these circumstances, the drug is quite effective against penicillin-resistant staphylococci. In the recent report of Finland and associates[17] it was concluded that bacitracin was less active in vitro against staphylococci than penicillin, streptomycin, aureomycin, and chloramphenicol.

Some consideration has been given to the possibility of employing an agent that would inhibit the action of penicillinase, and thus provide a means whereby penicillin could be administered effectively for infections with penicillin-resistant staphylococci that produce penicillinase. Perlstein and Liebman[51, 52] succeeded in producing an anti-penicillinase rabbit serum by injecting purified

penicillinase into the animals. This serum protected penicillin in vitro from destruction by the enzyme. These observations were later extended by Housewright and Henry.[53] It has been found more feasible in clinical practice to administer another antibiotic not affected by penicillinase.

In conclusion, penicillin cannot be employed today with a comfortable degree of confidence in the therapy of staphylococcic infections. In the more severe cases of staphylococcic sepsis it is recommended that as soon as possible in vitro sensitivity tests should be carried out with penicillin, streptomycin, aureomycin, chloramphenicol, and terramycin. A fairly dependable correlation exists between the results of the in vitro tests and the anticipated therapeutic activity of an antibiotic, provided a sufficiently large inoculum is used. If such tests are not immediately available, treatment with aureomycin will minimize the problem of resistance to antibiotic therapy.

SUMMARY

The incidence of strains of staphylococci which are highly resistant to penicillin is increasing at an alarming rate. This is emphasized from investigations at the University of Minnesota Hospitals. Prior to the introduction of penicillin in 1942, approximately 12 per cent of the strains of staphylococci isolated from patients were found to be naturally resistant to the drug. But over 50 per cent of the strains isolated in the latter part of 1949 and early months of 1950 were observed to be highly resistant to penicillin. This demonstration of penicillin resistance in vitro has been correlated with the therapeutic failures, and coincides with the experience of others.

From a clinical point of view the production of the enzyme penicillinase by resistant strains is quite important, and in vitro tests of penicillin sensitivity should take penicillinase into consideration.

Comparative in vitro tests with penicillin, streptomycin, aureomycin, chloramphenicol, and terramycin demonstrated that more strains of staphylococcus were sensitive to aureomycin than to each of the other antibiotics. Further clinical trials with chloramphenicol, terramycin, and combinations of antibiotics are desirable.

In the management of all serious staphylococcic infections, in vitro sensitivity tests against several antibiotics should be carried out, since an occasional strain may exhibit resistance to aureomycin.

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SOME FACTORS CONCERNED WITH THE TWO-STAGE PLASMA PROTHROMBIN ASSAY

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WARE and Seegers[1] have modified the original Iowa[2] two-stage method of plasma prothrombin assay in an endeavor to improve its accuracy and practical usefulness. It has long seemed to us[3] necessary to undertake certain experimental investigations, which have not appeared in the literature, in order to answer the question: Does the test truly measure *prothrombin* activity under the various conditions under which it may be used? In particular, the following studies aim to show (1) assay recovery of added purified prothrombin, (2) effects of heparin and (3) influence of fibrinolysin.

MATERIALS

1. *Human plasma* (H. Pl.): routinely prepared from arm vein blood of the same subject (C.L.J.) and containing (before centrifugation) one-tenth volume of 0.109M sodium citrate. In twenty-two successive tests on this citrated plasma, the prothrombin assay values averaged (range in parentheses), units per milliliter: (a) uncorrected, 231 (206 to 253); (b) corrected for hematocrit, 307 (274 to 339). Somewhat lower values (uncorrected, 200 ± 12 units per milliliter) were regularly obtained in some earlier tests and are validated for the comparative studies in which they appear.

2. *Purified bovine prothrombin* (Pro.): 0.02 per cent saline solution of a dry powder, kindly supplied by Dr. A. G. Ware and Dr. W. H. Seegers, who found it to contain 18,000 units per milligram tyrosine in original tests a year earlier (see text).

3. *Heparin* (Hep.): Sodium heparinate (Liquaemin, Roche-Organon), 1 c.c. = 10 mg. = 1,000 Toronto units; diluted with saline to give stock solutions of 100 and 10 units per milliliter respectively.

4. *Bovine serum fibrinolysin* (lysin): dry preparation, courtesy Dr. E. C. Loomis. Solutions 1 to 0.01 per cent freshly prepared as required.

5. *Thrombin* (Thr.): dry preparation, Parke Davis, courtesy Dr. E. A. Sharp, dissolved in glycerol (50 per cent)-saline to 100 units per milliliter.

6. *Thromboplastins:* Two of our own preparations (A and B) and two commercial products (C and D) were used, viz *Tpln. A*, from perfused ox lung, diluted 1:6; *Tpln. B*, from unperfused ox lung, diluted 1:6; *Tpln. C*, from rabbit lung (Squibb); *Tpln. D*, from rabbit brain (Difco). In comparative tests, these all gave indentical prothrombin unitage both on plasma and on purified prothrombin solutions, although the optimal incubation times varied, especially with Tpln. A which required about twice as long as the others.

7. *Bovine serum* (diluted): for supplying "accelerator factor(s)" in modified two-stage tests, was tried at several dilutions and 1:300 was found optimal for use in the incubation mix.

8. *Bovine fibrinogen(s)*: F_1: a "*cold* fibrinogen" from oxalated beef plasma, prepared according to Ware, Guest, and Seegers,[4] needed an additional step of adsorption with $BaSO_4$[5] in order to make it sufficiently free from prothrombin. As thus prepared, its solutions

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analyzed 1.8 to 1.5 per cent total protein and 85 to 90 per cent "clottable" (fibrin N) and were diluted to 1.0 per cent total protein for the assay tests. F_2: a 1 per cent borate buffer (pH = 7.7) solution of BF[6] (Armour's lyophilized bovine "fibrinogen"—Fraction I), tested on a number of occasions with H. Pl., gave prothrombin values ranging between 166 and 206 units per milliter (uncorrected), which were regarded as somewhat too low and too variable to be satisfactory for routine assays.[1] F_3: after adsorbing F_2 with $BaSO_4$ to free from all traces of prothrombin, and since thrombin and fibrinolysin were already absent, this fibrinogen was excellent for testing even minute quantities of (1) thrombin, (2) prothrombin, (3) antithrombin, (4) fibrinolysin, in such reagents as (a) serum, (b) thromboplastin, and so on (see below).

9. *Borate buffer solution*[6]: pH = 7.7, specific resistance 170 ohms (at 21° C.), effective ionic strength 0.055, contained (per liter): 11.25 Gm. H_3BO_3, 4 Gm. $Na_2B_4O_7$, $12H_2O$, 2.25 Gm. NaCl. In these studies it was only used in control tests on certain reagents (see text).

10. *Other reagents*: Imidazole buffer (pH : 7.25 ± 0.05); saline (0.9 per cent NaCl); $CaCl_2$ (0.67 per cent)—NaCl (0.4 per cent)—giving optimal conversion of prothrombin to thrombin at 0.010M Ca —; acacia (*purified,* free from Ca and oxalate and with less than 0.4 per cent NaCl): met the standards specified.

CONTROL OF REAGENTS AND METHODS

Exacting controls of all reagents and steps in the standardized procedures were carried out according to the recommendations of Ware and Seegers,[1] with the minimal requirements always exceeded and particularly so in the case of fibrinogen, thromboplastin, serum, and acacia. *Optimal* concentrations, times, and conditions were carefully determined for Ca, thromboplastin, serum, plasma, or prothrombin, dilutions, etc. The respective *dilution factors* at the successive steps of the assay were as follows: I, preparation of (citrated) plasma: 0 (uncorrected); 1.2-1.3 × (hematocrit correction); II, defibrination with thrombin: 2 ×; III, dilution with saline (original method)[2] or with serum-saline (modified method)[1]: 15-20 ×; IV, conversion of prothrombin to thrombin by optimal incubation, at 28°, with Ca-NaCl, thromboplastin; acacia, imidazole buffer: 4 ×; V, final timing of test clots, at 28° C, with fibrinogen (F_1): 1.25 ×.

Serum (1:150 dilution): showed (1) calcium—faint trace of turbidity with standard oxalate, (2) thrombin—mere trace of clot in F_3 in 30 min., (3) prothrombin—trace clot in mixture with Ca, thromboplastin, F_3, in 35 min., (4) antithrombin—increased clotting time of F_3 by 0.5 unit per milliliter thrombin from 100 sec. to 116 sec., (5) fibrinolysin—no clot lysis in 72 hr. at 37° C.

Thromboplastin (*Tpln. A,* in 1:6 dilution): showed (1) calcium—not demonstrable, (2) thrombin—none, (3) prothrombin—after incubation with standard incubation mix with saline instead of plasma, tract clot in F_1 in 35 min., (4) antithrombin—increased clotting time of F_3 by 0.5 unit per milliliter thrombin from 100 sec. to 131 sec., (5) fibrinolysin—no clot lysis in 72 hr. at 37° C.

Since plasma and purified prothrombin assayed the same unitage with or without serum (1:300) and with varying thromboplastins (including the commercial preparations which contained no prothrombin), it was concluded that none of the foregoing trace impurities were of any significance in the assay tests.

Accuracy of assay: averaged within a 10 per cent limit of error in sufficiently large numbers of tests performed simultaneously and with nonsignificant experimental variations on a number of occasions.

ASSAY OF PURIFIED PROTHROMBIN AND ITS ASSAY RECOVERY WHEN ADDED TO PLASMA

The purified prothrombin (Pro.) used in these studies originally contained 18,000 units per milligram tyrosine, according to Ware and Seegers' analyses. Assuming tyrosine to be 10 per cent of the dry weight, our stock 0.02 per cent

solution should contain 360 units per milliliter. Found values, however, were always much lower than this, being about one-half in freshly prepared solutions and about one-third, on the average, in a number of samples stored frozen at -20° C.

Stability studies: were made by repeated analyses every few hours during the day of preparation of the stock solution and every few days thereafter over a couple of weeks, (a) at room temperature (25 ± 2° C), (b) at ordinary refrigerator temperature (3 ± 2° C), and (c) at -20° C. (deep freeze). Although the instability was much reduced, it was still significant at the coldest (-20° C.) temperatures, e.g. (c) 166 (frozen and rethawed, immediately after preparation of stock solution), 143 (thawed six hours later), 138 (thawed thirty hours later), 119 units per milliliter (thawed nine days later): separate samples.

"Spontaneous" conversion to thrombin: The freshly prepared stock solution of this prothrombin contained no demonstrable thrombin. After standing for two hours at room temperature, it still did not clot fibrinogen (F_3) in one hour, although analyses showed a loss of 34 units per milliliter. After standing sixteen days in the refrigerator, it showed enough thrombin to clot fibrinogen (F_3) in forty-six seconds. The foregoing experimental facts, obtained incidentally in the present studies, clearly confirm the experience of the Wayne University workers on the following points.

1. Highly purified prothrombin, kept in desiccator at room temperature for over a year in the present instance, loses about half its original potency.

2. Solutions are unstable to a degree depending on the temperature.

3. While thrombin does appear "spontaneously" and gradually in solutions, this fails to account for most of the loss in prothrombin activity, according to results of assays by the two-stage method.

The basic principle of the Iowa two-stage method (and its more recent modifications) is to subject thrombin or prothrombin (before activation) to a *variable dilution* in order to produce a standard clotting time of 15 seconds, at 28° C. and under the conditions specified. This thrombin *unit* is regarded as an "absolute" value. The basically different principle, upon which all our coagulation research during the past two decades has relied, is that a "relative" potency value of thrombin can be assigned to *varying clotting times,* under standardized conditions,[7] and, in the activation of prothrombin, quantitative significance is given to various activator and inhibitor factors only when the dilution of the prothrombin mixture is not varied. Even the proponents of the two-stage technique must return to this principle in evaluating the time factor in the activation process. In the matter of thrombin yield, however, the fundamental difference in technique is apparent. The two methods give inexplicably divergent results on the question of the fate of prothrombin solutions.

In an extensive study[6] on a number of Seegers' highly purified prothrombins, which were studied (activated, etc.) in relatively strong solutions (several thousands of units per milliliter), the following data were obtained repeatedly. (1) Whatever the conditions of activation, the shortest clotting-times reached (regarded in our work as evidence of "complete" conversion to thrombin, under

the prevailing conditions) were identical, and, furthermore, (2) were exceedingly stable over at least three or four weeks at room temperature. In particular, (3) "spontaneous" activation, even though it required several weeks at room temperature, or longer in the refrigerator, yielded a thrombin of identical potency ("shortest clotting time") as that obtained, in a matter of minutes, with optimal activation by calcium and thromboplastin.

At several conferences, Dr. H. P. Smith and Dr. W. H. Seegers have expressed the criticism that our clotting times (3 sec. to 5 sec.) may have been too short to permit the detection of perhaps considerable thrombin differences. This doesn't seem a valid criticism since we have no difficulty in increasing the clotting times in the final thrombin dilution tests. In Table I, for instance, a twofold dilution (50 per cent) doubled the clotting time. Table I illustrates very clearly that *all* the prothrombin "spontaneously" converts into a stable

TABLE I. PERCENTAGE DILUTION TESTS* ON THROMBIN FORMED FROM SAME PROTHROMBIN SOLUTION: (I) WITH CALCIUM AND THROMBOPLASTIN, (II) "SPONTANEOUSLY"

MIXTURE	100%	50%	25%	10%	5%	1%
I	4 sec.	8 sec.	11 sec.	22 sec.	40 sec.	84 sec.
II	4 sec.	8 sec.	11 sec.	22 sec.	42 sec.	102 sec.

*Clotting times (seconds), at 24° C., pH = 7.7 (borate buffer), for 0.75 ml. fibrinogen (F_3) + 0.25 ml. stated dilution (%) of thrombic mixtures (see text).

thrombin under the test conditions we use. Table I gives the clotting times (0.25 ml. mixture + 0.5 ml. F_3) of serial (percentage) dilutions of the two original mixtures: I, 2 ml. Pro. (2,640 units per milliliter* + 2 ml. borate buffer + 0.75 ml. Tpln. D + 0.25 ml. M/10 $CaCl_2$ fully activated (4 sec. clotting time in 15 min.), stable for more than one week, and dilutions with borate buffer (Table I) tested on the third day. II, 2 ml Pro. (same solution, kept nine months in refrigerator) + 3 ml. borate buffer, and dilutions as above (Table I). The similarity of the two series of tests is remarkable and must be taken to mean very close equivalence in terms of thrombic potency.

Optimal incubation time: Another point, clearly stated by the workers who have purified prothrombin, is that a significantly longer time, even under apparently optimal conditions with respect to known activators (Ca, thromboplastin, "accelerator" factor(s)), is required for maximal thrombin formation (shortest clotting time test) with purified prothrombin as compared with diluted plasma. This is confirmed in the present work (e.g. Table II), the optimal

TABLE II. ASSAY RECOVERY OF PURIFIED PROTHROMBIN (PRO.) ADDED TO PLASMA (H. PL.) BEFORE (I) AND AFTER (II) DEFIBRINATION, IN TWO-STAGE METHOD OF DETERMINING PROTHROMBIN ACTIVITY

	MIXTURE	INCUBATION TIME (MIN.)	TOTAL FOUND (UNITS/ML.)	TOTAL EXPECTED (UNITS/ML.)	% DEVIATION FROM EXPECTED
I	H. Pl. + Pro.	16	228	227	0
II	H. Pl. + Pro.	22	160	144	+11
III	H. Pl.	14	188	–	–
IV	Pro.	35	99	–	–

*Seegers' data: *original* potency.

incubation times for Pro. averaging two or three times those required for 1:200 plasma, the variations with individual types of thromboplastin preparations being relatively minor and in no wise affecting the unitage obtained. When prothrombin is added to plasma *before* defibrination the optimal incubation time is lengthened only a couple of minutes, whereas when the prothrombin is added *after* defibrination the time is lengthened appreciably, but is ten to fifteen minutes shorter than with purified prothrombin alone (Table II).

Assay recovery of purified prothrombin. Table II shows assay data, average of triplicate determinations, obtained by the modified two-stage procedure, on I, (0.5 ml.) H. Pl. + (0.4 ml.) Pro., added *before* defibrination with 0.1 ml. Thr.; II, 0.5 ml. H Pl. (1:2 dilution after usual defibrination) + 0.5 ml. Pro.; III, H. Pl.; IV, Pro. (0.02 per cent). Note that the assay recoveries are as expected in I and practically at the upper limit (10 per cent) of error in II. In some older (1941) observations,[3] employing the previously discussed dilution technique to measure "relative" thrombin concentrations, we obtained assay increments very close to the determined activity of crude prothrombin added to plasma which had been diluted 50 to 100 times.

EFFECTS OF ADDED HEPARIN

A. In "second phase" (thrombin-fibrinogen reaction): Using various amounts of heparin in the saline with which the fibrinogen (F_1: see reagents) was diluted to 1 per cent before testing the clotting times with the (approximately) 1 unit of thrombin from diluted-activated plasma processed by the usual modified two-stage method, we obtained the data summarized in Table III, which show how the presence of heparin (with such "cofactor" as is presumably supplied in the reagents) influences the clotting times and thrombin units obtained, the latter being calculated, as usual, per milliliter of original plasma (uncorrected). The smallest heparin concentration tested (0.067 unit per milliliter) definitely prolongs the clotting time and lessens the computed thrombin unitage.

TABLE III. EFFECTS OF VARIOUS CONCENTRATIONS OF HEPARIN UPON CLOTTING TIMES AND THROMBIN UNITS OBTAINED IN FINAL STEP OF THE TWO-STAGE ASSAY OF PLASMA PROTHROMBIN

	1	2	3	4	5	6
Heparin concentration (u/ml, final clotting mixt.)	0	0.134	0.26	0.4	0.66	6.6
Clotting time (sec.)	15.6	17.4	17.9	18.8	20.0	28.8
Estim. thrombin units (per ml. "original" plasma)	191	166	161	152	144	88

B. In "first phase" (activation of prothrombin to thrombin): *I. Heparin added AFTER defibrination.* In the data of Table IV, the heparin was added (a) with the serum-saline diluent in tests 2 to 5, (b) with the incubation mix in test 6, and (c) immediately after the defibrination in test 7. The least amount of heparin (0.02 unit per milliliter in thrombic mixture) had no significant effect, but all higher concentrations tested (0.168 to 1.25 units per milliliter) greatly prolonged the clotting times, caused clot of poor quality (flocculent and stringy), and made determination of the thrombin unitage impossible. The

optimal incubation periods were only slightly increased, however. It is particularly significant that 0.168 unit per milliliter heparin (in thrombic mixture of test 3) prevented assayable thrombin formation, although the second phase effects (Table III, test 2) are very minor.

TABLE IV. EFFECTS OF VARIOUS CONCENTRATIONS OF HEPARIN, ADDED AFTER DEFIBRINATION, UPON CLOTTING TIMES AND THROMBIN UNITAGE IN TWO-STAGE ASSAY OF PLASMA PROTHROMBIN

	1	2	3	4	5	6	7
Heparin concentration							
1. (u/ml. thrombin mixt.)	0	0.02	0.168	0.33	0.67	0.75	1.25
2. (u/ml. final clot mixt.)	0	0.016	0.134	0.26	0.536	0.6	1.0
Clotting time (sec.)	15.6	15.2	100	135	164	194	212
Estim. thrombin units (per ml. "original" plasma)	191	194	--	--	--	--	--

II. Heparin added to plasma BEFORE defibrination. When 0.5 ml. plasma was mixed with 0.4 ml. heparin (33 units per milliliter) before defibrinating with 0.1 ml. thrombin and carrying through the subsequent steps of the assay procedure, no thrombin was detectible in the incubation mixture even after four hours. The heparin concentration in the incubation mixture was 0.168 unit per milliliter, so this finding agrees with test 3, Table IV, in showing the great interference with the assay test chiefly due to heparin inhibition of the prothrombin activation. This amount of heparin, however, is much in excess of therapeutic concentrations such as may be encountered in the blood clinically. Loomis[8] considers 0.001 mg. (0.1 unit) per milliliter blood typical of the amounts usually required for anticoagulant therapy, and Jaques and Ricker[9] state that such concentration will increase the whole blood clotting time (Lee-White method) to about 20 to 25 minutes.

In the following experiments, repeated on twelve separate occasions, we approximated this therapeutic heparin concentration by adding 0.1 unit per milliliter heparin to the plasma during the 1:2 dilution immediately *before* defibrination, i.e., 0.5 ml H. Pl. + 0.4 ml. 0.25 unit per milliliter Hep. + 0.1 ml. Thr, the rest of the assay procedure being continued in the usual way. In each case, a parallel control was run without the heparin. The average unitages (range in parentheses) were as follows: 1, with heparin: 233 (224 to 240); 2, controls, without heparin: 201 (192 to 212). In four additional experiments, a similar amount of heparin was added *after* defibrination, giving the data 194 (184 to 202), which were not significantly different from the foregoing data for the controls, thus proving that this small concentration of heparin is without any effects on the first or second phases of the clotting mechanism. The significant *increase* in unitage in all the tests (1) on heparinized plasma is wholly unexpected and poses new and difficult problems. The following experiments offer a number of points, most of them negative, in weighing the evidence thus far available.

With the small amount of heparin present, the defibrination clotting time by the 10 unit thrombin (per milliliter mixture of step II) was only slightly lengthened, 19 sec. instead of 15 sec., i.e., just enough to indicate that the heparin

was adding some antithrombic value. If anything, this might be expected to enhance the rate of disappearance of the added thrombin after the defibrination. Actual tests of the defibrinated plasmas (with and without heparin), 0.2 ml. sample added to 0.2 ml. 1 per cent fibrinogen, after the usual ten minutes allowed for the antithrombins to inactivate the added thrombin, gave clotting times longer than 20 min. in both cases. Thus, there is no a priori reason or experimental observation to suggest any persistence of the thrombin used for defibrination.

III. Experiments with purified prothrombin solution. Two-stage (modified) prothrombin assays were performed on mixtures of 0.5 ml. Pro. (0.02 per cent) with (1) saline, (2) thrombin, (3) thrombin and fibrinogen, (4) thrombin, fibrinogen, and heparin. Table V gives details of these mixtures and the assay data. Note the following: (1) The artificial addition of thrombin-fibrinogen (with subsequent defibrination) *increases* the assay unitage especially significantly when heparin is present, in a manner apparently similar to the observations of the plasma experiments of the preceding section. The small amount of thrombin added is readily demonstrable in the special clotting tests of the last column (Table V) in which 0.2 ml. of mixture (after defibrination in tests 3 and 4) was added to 0.2 ml. 1 per cent fibrinogen. There being no antithrombin present, the slight weakening of the thrombin after simple defibrination (test 3) and more marked loss after defibrination in the presence of heparin are probably due to adsorption loss on the fibrin clot.[10]

TABLE V. ASSAY OF PURIFIED PROTHROMBIN IN PRESENCE OF THROMBIN, FIBRINOGEN, HEPARIN

TEST	PRO. (81 U/ML.) ML.	THR. (50 U/ML.) ML.	HEP. (0.5 U/ML.) ML.	F_1 (1.5%) ML.	SALINE (0.9% NACL) ML.	ASSAY UNITS* (PER ML. "ORIGINAL" (PRO.)	THROMBIN TEST C.T. (SEC.)†
1	0.5	–	–	–	0.5	81	–
2	0.5	0.1	–	–	0.4	80	12
3	0.5	0.1	–	0.2	0.2	89	15
4	0.5	0.1	0.2	0.2	–	100	20

*At final dilution 1:100.

†0.2 mixt. (after defibrination in tests 3 and 4) + 0.2 ml. 1 per cent F_1: clotting time, seconds, at 28° C.

That the added thrombin is diluted out and is not enough to affect the assay values is evident from the similarity of tests 1 and 2. Considering the possibility (Ware and Seegers[11]) that the thrombin might possibly be gradually destroying some of the prothrombin, test 2 was repeated with a sixty-minute incubation of the prothrombin and thrombin. Exactly the same unitage (80 units per milliliter) was subsequently found, so that this possibility seems ruled out in the present experiments, as it must, of course, be in all valid plasma prothrombin assays. Testing the undiluted mixtures, after defibrination, with thrombin proved the defibrination to be complete, and the fact that the unitage in test 3 was slightly higher than in test 1 shows that no prothrombin could have been lost by adsorption onto the fibrin clot.

EFFECTS OF ADDED FIBRINOLYSIN

A potent preparation of bovine serum fibrinolysin (*lysin*—see reagents)[12] was tested in very much the same manner as heparin in the preceding section of these studies.

A. Effects of fibrinolysin in "second phase." Fibrinogen (F_1) was diluted to 1.0 per cent with saline solution containing 0.1 per cent lysin, to give a lysin concentration of 0.33 mg./per milliliter of fibrinogen and one-fifth of this in the final clotting mixture, containing approximately 1 unit of thrombin activated in the usual way in defibrinated diluted human plasma. In eight tests (1) with fibrinolysin: the assay units were 219 (182 to 238); (2) without fibrinolysin: 222 (206 to 243), i.e. not significantly different.

The enzyme was added only a few minutes before the test so that there was no significant fibrinogenolysis. Clot lysis was not timed but was a matter of several hours.

B. Effects of fibrinolysin in "first phase." Running several (three to five) experiments in each series, fibrinolysin (0.0067, 0.0075, 0.075 mg./per milliliter incubation mixture) was added (a) immediately *after* defibrination, (b) with the serum-saline diluent, or (c) with the incubation mix. The optimal incubation times were not significantly altered and the assay values, 245 ± 20 units per milliliter, were practically the same as controls (without fibrinolysin), 230 ± 15, within probable limits of experimental error.

C. Effects of adding fibrinolysin to plasma. 0.4 ml. 0.75 mg./per milliliter fibrinolysin added to 0.5 ml. plasma *before* defibrination was equivalent to 0.0075 mg./per milliliter lysin in the incubation mixture. Assays (1) with the fibrinolysin averaged 238 units per milliliter, and (2) controls, without enzyme, 235 units per milliliter, i.e. practically identical values.

CONCLUSIONS

With sufficient experience in the preparation and testing of reagents, controlling the latter for possibly significant impurities, and determining optimal concentrations, times, and conditions at each of the several steps, the two-stage method of plasma prothrombin assay, in either the original[2] or modified[1] form, gives reliable results within an experimental error of some 10 per cent.

Purified prothrombin, freshy assayed for actual unitage at the time of tests, can be quantitatively "recovered" in assays made when the prothrombin is added to plasma either before or after defibrination.

Heparin in "unphysiologic" concentrations reduces the assay values when added to the final clotting mixture, and when added at any step during the dilution (after defibrination) and activation of the plasma, prevents assayable quantities of thrombin from forming, even when the heparin concentration is reduced so as to have very little "second phase" effect. In therapeutic concentrations, corresponding to about 0.1 unit per milliliter whole blood, heparin does not exert any inhibitory effects but, when added to plasma *before* defibrination, gives a significant elevation of the assay values.

By finding a similar phenomenon when purified prothrombin is artificially treated with thrombin-fibrinogen and defibrinated, with or without these small amounts of heparin, we conclude that this anomalous phenomenon depends in some way upon the defibrination. However, it is not explained in any simple manner.

Small amounts of fibrinolysin do not significantly modify the plasma prothrombin determinations.

The two-stage technique gives data concerning the stability of prothrombin solutions and particularly on the yields of thrombin resulting from "spontaneous" activation which are not in agreement with the old method of determining "relative" thrombic potency in terms of varying clotting times. The validity of the latter is supported by data given in Table I.

In summary, it is concluded that there is still a lot to be learned about the true significance of the "units" obtainable by the two-stage method under a variety of circumstances of experimental and practical significance. The ability to obtain good assay "recoveries" of added purified prothrombin is the best evidence that the test is usually a reliable measure of plasma prothrombin activity.

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ENZYME STUDIES ON HUMAN BLOOD

VIII. The Effect of Fibrinogen Concentration on the Thrombin Clotting Time

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THE determination of fibrinogen is of importance in the investigation of not only the coagulation mechanism but also of other physiologic and pathologic processes. The methods used chiefly in recent years have been those of Howe,[1] Cullen and Van Slyke,[2] Gram,[3] Foster and Whipple,[4] and Wu.[5] An analytic principle finding increasing use is electrophoretic resolution[6, 7] of whole plasma coupled with Kjeldahl nitrogen determination.

With the development of the low temperature-low ionic strength principle by Cohn and co-workers,[8] a new basis for the separation of fibrinogen as Fraction I was made available. Edsall and associates[9] have investigated Fraction I obtained from pooled plasma with electrophoresis and by thrombin-clottable protein determinations. More recently, Pearsall and Chanutin[10] have analyzed with electrophoresis and Kjeldahl whole plasma and Fraction I prepared from 20 ml. samples of normal plasma with a modified low temperature procedure. They reported their results as the ϕ component in Fraction I and in other fractions.

Previous work[11] in this laboratory indicated the possibility that fibrinogen in Fraction I could be estimated by thrombin clotting time determinations at concentrations below the optimum for the substrate. It was also shown that plasma proteins have a marked influence on the thrombin-fibrinogen reaction.[12] The determination of fibrinogen on a fraction low in other plasma proteins and obtainable from a small specimen was made possible by the development of the new homologous isolation procedure for prothrombin.[13] In the first stage of this technique fibrinogen is separated out quantitatively as Fraction I by Method 6 of Cohn and associates[8] applied to 4.0 ml. of citrated plasma. This communication reports the plasma fibrinogen concentration levels derived by clotting time determinations of diluted Fraction I solutions and standard thrombin reagent. These results are compared with those obtained by the determination of thrombin-clottable protein[9] on a total of 139 patients of the University Hospital and 59 students and faculty members of the College of Medicine.

METHODS AND MATERIALS

Preliminary Treatment of Fraction I.—The collection of blood and the separation of Fraction I from 4.0 ml. of citrated plasma have been described in detail in a previous communication.[13] After obtaining the Fraction I precipitate, exactly 4.0 ml. of 0° citrate-phosphate

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buffer Γ/2 (ionic strength) 0.129, pH 7.2[13] was added without delay. The mixture was permitted to warm to room temperature with occasional gentle stirring. After twenty to thirty minutes the Fraction I solution was centrifuged for twenty minutes, 900 × g, room temperature, to remove traces of insoluble protein, if present. The supernatant solution was then decanted into a clean test tube.

The Determination of Fibrinogen by Clotting Time.—To 0.8 ml. of Fraction I diluted with citrate-phosphate buffer was added 0.2 ml. of a standard thrombin reagent, 5.0 units per milliliter, and the clotting time was determined in duplicate or triplicate as previously described.[13] For most determinations 1:10 or 1:20 dilutions of Fraction I solution were tested. In cases of hypo- or hyperfibrinogenemia, lesser or greater dilutions were made. In all cases dilutions were chosen to give a clotting time between 20 and 30 seconds. The standard thrombin reagent was checked daily against fibrinogen solutions at optimum concentrations. The following equation was employed for the calculation: $1/k\ t^2 \times D \times C =$ per cent plasma fibrinogen, where C is the citrate dilution of plasma; and in reference to the diluted Fraction I, D is the dilution, t is the mean clotting time in seconds, and k is 0.0975. The foregoing equation was simplified as follows:

$$\frac{10.2564}{t^2} \times D \times C = \text{per cent plasma fibrinogen.}$$

The mean k factor should be determined at several concentration levels of fibrinogen by each laboratory.

Estimation of Thrombin-Clottable Protein.—Into a 15 ml. centrifuge tube were pipetted, in order, 2.0 ml. of thrombin, 2 units per milliliter, 6.0 ml. of citrate-phosphate buffer, and 2.0 ml. of the Fraction I solution. The contents were mixed by inversion and allowed to stand for two hours in a 37.5° water bath. The fibrin was loosened with an applicator stick and then removed by centrifugation for fifteen minutes at room temperature and 900 × g. Protein determinations were done by chemical and/or physical procedures on the supernatant solution (nonclottable protein), on a 1:10 dilution of the Fraction I solution (total protein), and on a 1:5 dilution of the thrombin reagent (blank).

The tyrosine equivalents of the foregoing solutions were determined by the method of Folin and Ciocalteu.[14, 15] Readings of per cent transmission were made on the Coleman Junior spectrophotometer at 650 mμ, five minutes after the addition of the phenol reagent. Generally 4.0 ml. aliquots of diluted protein solutions as obtained above were tested. The following equation was employed for the calculation: $\text{Gm.} \times \frac{100 \text{ ml.}}{V} \times 16 \times C =$ per cent protein where Gm. refers to the actual grams tyrosine found colorimetrically; V is the actual volume in milliliters of the original protein solution tested; 16 is the chromogenic factor for Fraction I in regard to chemically pure tyrosine; and C is the correction for the citrate dilution of plasma. Tyrosine values for nonclottable protein were corrected for the thrombin reagent blank. Thus the fibrinogen cencentration was found by subtracting the per cent nonclottable protein from the per cent total protein in Fraction I.

The protein concentrations of the various solutions already described were also determined directly by ultraviolet absorption. The optical densities of the diluted Fraction I, the nonclottable portion of Fraction I, and the thrombin reagent were read directly against citrate-phosphate buffer reference at 280 mμ on the Beckman quartz spectrophotometer with ultraviolet accessory (Model D.U). The following equation was employed for the calculation:

$$\left[\left(\frac{d\ T}{12.77} \times 10\right) - \left(\frac{d\ NC - d\ THR}{11.71} \times 5\right)\right] \times C = \text{per cent fibrinogen,}$$

where d T = density of the total protein solution; 12.77 is the K factor of the total protein solution; d NC = density of the nonclottable protein solution; d THR = density of the

thrombin reagent blank; 11.71 = K factor for the nonclottable protein solution; 10 is the dilution of the total protein solution; 5 is the dilution of the nonclottable protein solution; and C is the correction for the citrate dilution of plasma.

RESULTS

The tyrosine equivalents of Fraction I and the nonclottable protein of this fraction were determined directly on protein solutions of known concentrations established gravimetrically rather than by Kjeldahl nitrogen determinations. Fraction I was prepared from 2 liter lots of surplus human plasma by Method 6 of Cohn and co-workers.[8] The Fraction I precipitate was lyophilized, initial temperature –25° C., and pressure 30 micra mercury, and then stored in vacuum over silica gel. A 1.0 per cent suspension of Fraction I in citrate-phosphate buffer was heated at 56° C. for five minutes. After centrifugation the supernatant fluid was lyophilized. For the colorimetric determinations, 0.10 per cent (by weight) suspensions in citrate-phosphate buffer of fresh Fraction I and of nonclottable protein were prepared. The latter was corrected for salt content. The chromogenic factor in respect to chemically pure tyrosine as determined by the Folin-Ciocalteu[14, 15] reagent was found to be 16.03± S.D. 0.74 for Fraction I (ten experiments), and for the nonclottable portion of Fraction I, 15.96± S.D. 0.88 (five experiments). Wu[5] stated that 1 mg. tyrosine is equivalent to 16.4 mg. fibrin. Andersch and Gibson[16] reported a tyrosine chromogenic factor of 13.0 for fibrinogen.

The completeness of removal of fibrinogen as Fraction I by the low temperature method[8] as done in this laboratory on 4.0 ml. of citrated plasma was determined. In 348 consecutive fractionations, the fibrinogen concentration in the pH 5.05 prothrombin fraction,[13] which is removed from the plasma after the Fraction I, was found to range between 0.00 and 0.01 per cent with a mean of 0.004 per cent. This amount represented less than 1.5 per cent of the total plasma fibrinogen. In other words, more than 98 per cent of the plasma fibrinogen was removed in Fraction I. Subsequently, this recovery has been found to be even greater if the alcohol-plasma mixture is stirred thirty minutes rather than "at least fifteen additional minutes" as previously reported.[13]

Fig. 1 represents clotting time determinations on 285 solutions of Fraction I obtained from normal and pathologic individuals. Each determination is the average of at least three clotting time tests. Each experimental value is the composite of results obtained on four to thirty-three diluted Fraction I solutions of known clottable protein concentration as determined chemically. The relationship between per cent fibrinogen concentration (c) and clotting time in seconds (t) can be expressed by the equation $k = 1/c\,t^2$ (curve, Fig. 1). This constant (k) determined from 145 diluted Fraction I solutions of known fibrinogen concentrations between 0.012 and 0.025 per cent and clotting between 20 and 30 seconds was found to be 0.0975. Between the foregoing limits it is evident that this theoretical curve coincides with the experimental values. Above 30 seconds clotting time, deviation from theory can possibly be explained on the basis that the fibrinogen concentration is of such low magnitude that the

endpoint is difficult to observe accurately. Below 20 seconds clotting time there is also deviation from theory since the concentration is approaching the optimum (0.1 to 0.3 per cent) for the thrombin-fibrinogen reaction.[11]

Concomitant fibrinogen results obtained by clotting time and by chemical means on Fraction I solutions from 145 normal and pathologic individuals are shown in the scatter diagram, Fig. 2. In the former technique Fraction I was diluted to give a clotting time between 20 and 30 seconds. When clotting times at two dilutions fell in this range the protein concentration values obtained were averaged. In more than two-thirds of the 145 specimens the maximum deviation in the fibrinogen concentrations obtained by the two techniques was 10 per cent.

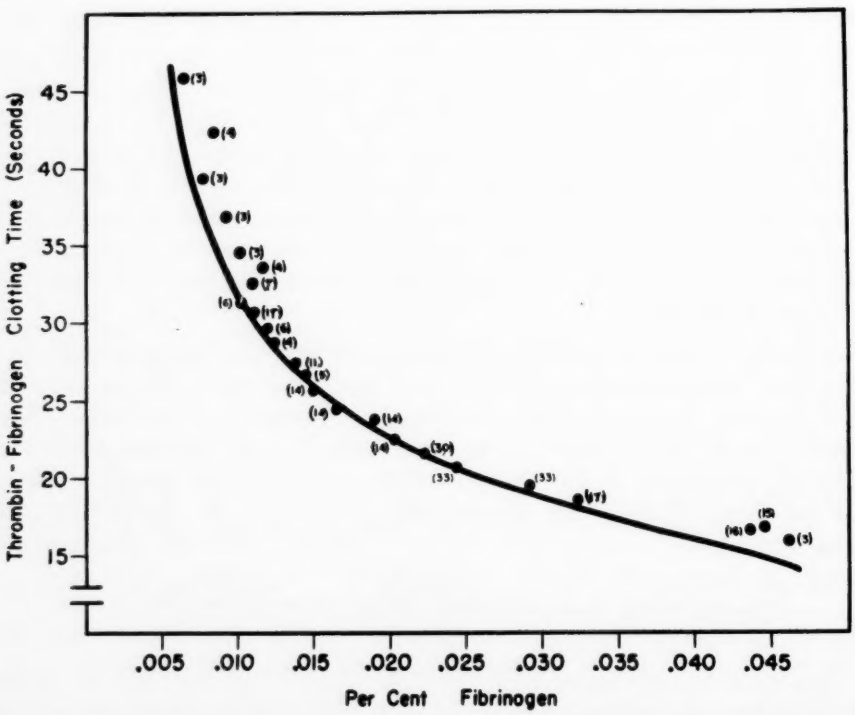

Fig. 1.—The effect of fibrinogen concentration on the thrombin clotting time. The dot represents the mean clotting time of several Fraction I solutions; the figure in parentheses indicates the number of solutions tested; and the curve is the theory or $k = 1/c\ t^2$. Standard thrombin, 0.2 ml., 5 units per milliliter added to 0.8 ml. fibrinogen solution. $\Gamma/2$, 0.129; pH 7.2.

Ultraviolet absorption studies on Fraction I and on the nonclottable subfraction showed a maximum extinction at 280 mμ and a minimum extinction at 254 mμ. Kunitz[17] found that a typical protein has a maximum extinction at 280 mμ and a minimum extinction at 252 mμ. Density (d) measurements were made on 139 solutions of nonlipemic Fraction I and 133 solutions of nonclottable subfractions in which the actual protein concentrations (c) were determined chemically. K factors were then calculated according to the equation $K = d/c$, and are recorded in Table I. The density readings at 280 mμ show

TABLE I. THE RELATIONSHIP (K) OF OPTICAL DENSITY (d) AND TOTAL AND NONCLOTTABLE PROTEIN CONCENTRATIONS (c) OF FRACTION I AT 280 mμ AND 254 mμ

WAVE LENGTH (Mμ)	K = d/c							
	TOTAL PROTEIN				NONCLOTTABLE PROTEIN*			
	n	MEAN	±S.D.	C.V.%	n	MEAN	±S.D.	C.V.%
280	139	12.77	0.677	5.30	133	11.71	0.974	8.32
254	139	5.76	0.555	9.63	133	5.49	0.735	13.20

*Corrected for thrombin blank.

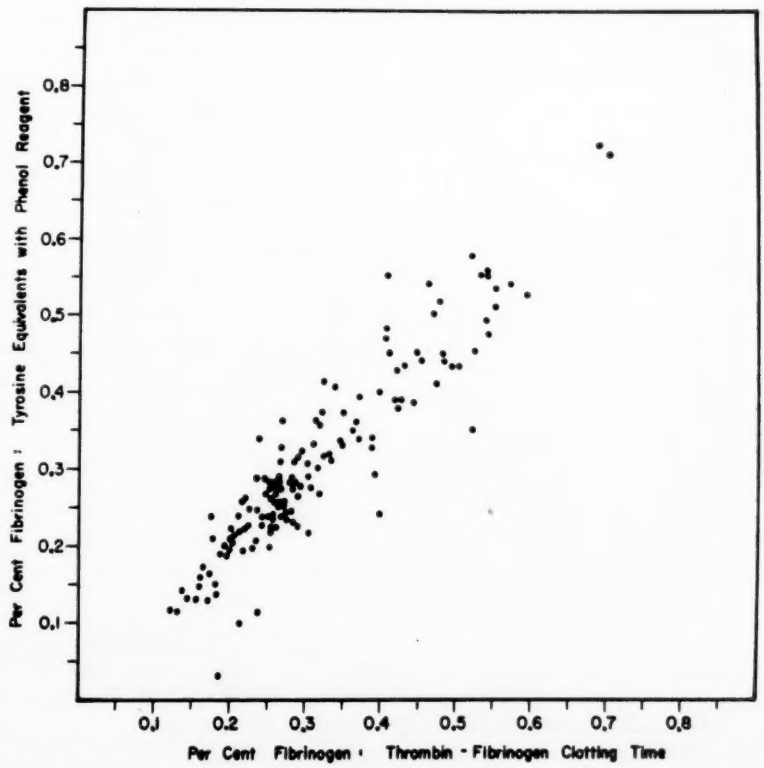

Fig. 2.—Scatterdiagram of plasma fibrinogen concentrations by clotting time and thrombin-clottable protein determinations in 145 individuals.

a greater sensitivity per milligram of protein concentration than those at 254 mμ. The concomitant results on 129 samples obtained by chemical determination and by ultraviolet absorption at 280 mμ are shown in the scatter diagram, Fig. 3. In more than two-thirds of the 129 specimens the maximum deviation in the fibrinogen concentration obtained by the two techniques was 10 per cent.

The mean plasma fibrinogen content of healthy, nonhospitalized, male and female adults, 20 to 60 years old, by clotting time and thrombin-clottable protein determinations are shown in Table II. The results by the various methods were almost identical.

TABLE II. NORMAL PLASMA FIBRINOGEN VALUES AS DETERMINED BY TWO METHODS

METHOD		n	FIBRINOGEN CONCENTRATION MEAN %	±S.D. %	C.V. %
Thrombin-clottable	Chemical	59	0.2636	0.0490	18.58
	Ultraviolet	26	0.2632	0.0421	15.99
Clotting time		37	0.2666	0.0367	13.77

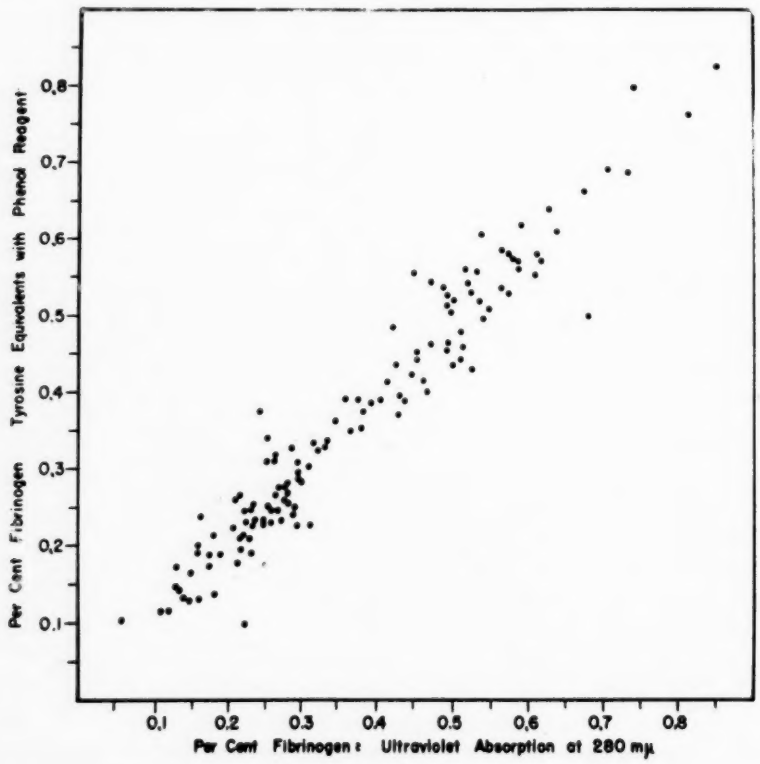

Fig. 3.—Scatterdiagram of plasma fibrinogen concentrations determined chemically and physically in 129 individuals.

DISCUSSION

The results in the present work demonstrate primarily that the clotting time in seconds (t) of 0.2 ml. standard thrombin, 5 units per milliliter, and 0.8 ml. human Fraction I[8] solutions of less than 0.025 per cent fibrinogen concentration (c) can be expressed by the equation: $k = 1/c\,t^2$. This finding has had several practical applications in this laboratory. It has served as a basis for a simple and rapid determination of fibrinogen concentrations. Moreover, in a few patients to be reported elsewhere, there was apparently afibrinogenemia by the determination of thrombin-clottable protein. However, by the clotting time technique, it was found that fibrinogen in a low concentration was actually present. This discrepancy in these cases was explained by the observation that the clot dissolved in less than thirty minutes after its formation.

The mean normal fibrinogen concentration of 0.26 per cent found in the present study is in agreement with the observations of other workers[3, 18-22] who with phenol reagent or Kjeldahl determined the fibrin obtained by the addition of calcium chloride to whole plasma. Significantly lower values were found by Trevorrow and co-workers[23] who employed sodium sulfate to separate fibrinogen. Considerably higher levels have been reported by those employing electrophoresis and Kjeldahl on whole plasma [10, 24-27] and on Fraction I.[10, 27] Thus, electrophoretic and Kjeldahl data of Pearsall and Chanutin[10] indicate the following normal fibrinogen concentrations: Fraction I, 0.266 per cent; all fractions, 0.642 per cent; and whole plasma, 0.474 per cent. The foregoing values were calculated by the present authors from the ϕ component and with the assumption that protein contains 16 per cent nitrogen. The higher values obtained by electrophoresis are attributed by Edsall and associates[9] to the incomplete resolution of the fibrinogen peak from the slowest components in the β-globulins. These authors found the mean fibrinogen concentration to be 0.28 per cent in twelve pooled normal plasma samples; Kjeldahl determinations were done on fibrin obtained by thrombin.

SUMMARY

Fraction I was obtained from 198 normal and pathologic individuals by the low temperature-low ionic strength method as applied to 4.0 ml. samples of plasma. It was found that at pH 7.2 and $\Gamma/2$ 0.129, the clotting time in seconds (t) of 0.2 ml. of standard thrombin, 5 units per milliliter, and 0.8 ml. diluted Fraction I solutions of less than 0.025 per cent fibrinogen concentration (c) can be expressed by the equation: $k = 1/c\,t^2$. This relationship of a variable concentration of substrate with a constant potency of enzyme is the basis for a simple and rapid determination of fibrinogen. The results by this method are compared with those obtained by thrombin-clottable protein determination. The mean fibrinogen concentration of fifty-nine normal individuals was found to be 0.26 per cent ± S.D. 0.05 per cent.

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ENZYME STUDIES ON HUMAN BLOOD

IX. The Fibrinogen Content of Stored Plasma

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DESPITE the importance of fibrinogen, the quantitative study of its stability in stored human plasma has received scant attention. Sterile citrated or oxalated plasma standing at 4° C. has a tendency to form a precipitate[1, 2, 3] believed by some[4, 5, 6] to be fibrin and to be fibrinogen by others.[1, 7] Flocculation reaches a maximum in a few hours, according to Strumia and McGraw,[3] while White and Weinstein[5] state that it begins in seven to ten days and increases rapidly throughout the first few months of preservation. At room temperature flocculation is almost eliminated[3] or lessened, and most frequently seen in one to three months of storage.[5] Taylor and associates[8] report normal fibrinogen after six months' storage of citrated plasma at room temperature, "when it was possible to determine it"; while it could not be determined in the older liquid plasmas because of failure of the clotting mechanism. Lipp and Hubbard[9] report increases in fibrinogen from 0.26 to 0.55 per cent and from 0.195 to 0.39 per cent in the plasma of citrated blood stored aseptically at 4° C. up to thirty-six days. Lord and Pastore[10] report no significant change in average fibrinogen levels in three weeks of storage of bank blood at refrigerator temperature. Electrophoretic analysis by Krejci and co-workers[11] of four samples of sterile pooled liquid plasmas stored at room temperature for three years showed a complete absence of fibrinogen. However, Scudder[12] by electrophoretic determinations showed no difference in ϕ component in twelve citrated bank plasmas refrigerated for twelve to fifty-three days and in three samples of fresh normal citrated plasma.

Some investigations on prothrombin stability in plasma have included observations on qualitative changes in fibrinogen. Thus Banfi and co-workers[4] attribute the noncoagulability of plasma stored five months at refrigerator temperature to an alteration or disappearance of the fibrinogen. Loomis and Seegers[13] have postulated that fibrinogen is denatured during refrigerator storage of human plasma and its decreased reactivity was responsible for the delayed prothrombin clotting times by the one-stage technique. Alexander and DeVries[14] claim that the fibrinogen of stored plasma becomes progressively less coagulable. In contrast to the previous investigators, Quick[15] states that fibrinogen is not appreciably altered in activity in plasma stored for forty-eight hours, while Honorato[16] believes this protein on storage becomes more easily coagulable.

For some time in this laboratory Fraction I has been prepared from large volumes of stored plasma by the low temperature-low ionic strength method of Cohn and associates.[17] In addition, methods for the determination of fibrinogen

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in Fraction I obtained from small volumes of plasma have been developed.[18] Therefore, in view of the discrepancies in the status of fibrinogen in stored plasma, this communication presents preparative and analytic data on six samples of liquid plasma stored up to seven and a half months, one frozen sample stored for fifteen months, and thirteen dried samples stored from forty-eight to seventy-two months.

METHODS AND MATERIALS

Liquid Plasma.—The aged liquid samples were prepared by the University Hospital blood bank in routine manner; plasma from 8 units of citrated whole blood stored approximately two weeks at 3 to 5° C. was pooled and maintained at the same temperature for four to seven and a half months. Prior to the preparation of Fraction I, the stored liquid plasma was filtered through gauze to remove any fibrin veils or other gross particulate matter which invariably appears during storage.[1-7]

Frozen Plasma.—Approximately 500 ml. of blood were collected by the blood bank from each of four normal adult male subjects. The sterile citrated whole blood was then brought to this laboratory, where centrifugation at +1° C. was begun within thirty minutes after venipuncture. The four samples of plasma were then pooled and Fraction I was prepared according to Method 6 of Cohn and co-workers.[17] A sample of the pooled sample was stored in a -25° C. refrigerator for fifteen months.

Dried Plasma.—The starting material was surplus Army-Navy normal human dried plasma as collected by the American Red Cross and processed by private concerns. The age of each preparation was calculated from the five-year expiration date. The dried plasma was reconstituted with 0.1 per cent citric acid to the volume of the "original normal plasma" as indicated on the container.

Preparatin of Fraction I.—Fraction I was prepared from 2 liter lots of plasma according to the method of Cohn and associates.[17] Fraction I was lyophilized, begining temperature -25° C., pressure 30 micra. The dry powder was weighed and stored in a vacuum desiccator over silica gel. A 10 per cent suspension by weight in citrate-phosphate buffer, $\Gamma/2$, 0.129, pH 7.2, was stirred for fifteen to twenty minutes and then centrifuged, 900 × g, to remove traces of insoluble material. The supernatant was then analyzed for total protein and thrombin-clottable protein.[18] Thus the fibrinogen content of stored plasma by the preparative technique was calculated from the yield, solubility, and the per cent thrombin-clottable protein of Fraction I obtained from large volumes of sample.

Analytical Determination of Fibrinogen.—Fibrinogen was also determined directly on 4.0 ml. samples of stored plasma by the chemical method recently described.[18]

RESULTS

In Table I are found the average fibrinogen contents, determined by two procedures, of liquid, frozen, and dried normal plasma stored for varying lengths of time and temperature. They are compared with the values for fresh normal plasma previously reported.[18] The values obtained by the preparative procedure were calculated from the data on Fraction I isolated from 2 liter lots of pooled plasma. They are in excellent agreement with those obtained directly by the 4.0 ml. analytic technique.

The six samples of pooled liquid plasma stored an average of 5.6 months at 3 to 5° C. had a mean fibrinogen content comparable to that found for fresh plasma. There was no correlation between the fibrinogen content and the age in this group of samples, which varied from four to seven and a half months. In one experiment Fraction I was prepared from 1 liter of plasma obtained

TABLE I. THE FIBRINOGEN CONTENT OF STORED PLASMA IN LIQUID, FROZEN, AND DRIED FORMS

TYPE OF PLASMA	STORAGE		NUMBER OF SAMPLES	PERSONS PER SAMPLE	FRACTION I			PLASMA FIBRINOGEN	
	AGE	TEMPERATURE (°C.)			YIELD (GM./L.)	SOLUBILITY (%)	THROMBIN-CLOTTABLE PROTEIN (%)	PREPARATIVE TECHNIQUE (%)	ANALYTIC TECHNIQUE (%)
Liquid	15 min.	0-1	59*	1	—	—	—	—	0.2636 S.D. ± 0.0490
	60 min.	0-1	1	4	4.16	90.0	60.9	0.2280	
	5.6 mo. S.D. ± 1.5	3-5	6	8	4.62 S.D. ± 0.20	87.5 S.D. ± 6.0	62.1 S.D. ± 1.9	0.2510 S.D. ± 0.0217	0.2438 S.D. ± 0.0258
Frozen	15 mo.	-25	1	4					0.2385
Dried†	60 mo. S.D. ± 12	Variable	13	8	4.95 S.D. ± 0.50	93.4 S.D. ± 6.2	63.1 S.D. ± 2.0	0.2941 S.D. ± 0.0292	0.2849 S.D. ± 0.0320

*From a previous report.[18]

†Each unit represents more than eight individuals and was stored at variable temperatures.

from four individuals and which was less than sixty minutes old. The data on Fraction I indicated a fibrinogen content of 0.2280 per cent. A small portion of this same plasma was stored at –25° C. for fifteen months, at which time the fibrinogen content was 0.2385 per cent by the analytic technique. The mean fibrinogen content of the thirteen dried plasma samples was not significantly different from that of the mean value of fresh normal plasma.

DISCUSSION

In general, the results in the present study demonstrate that fibrinogen is stable in liquid, frozen, and dried plasma for at least the periods of time indicated. There is no significant difference in the fibrinogen content of these stored samples and of fresh normal plasma. Qualitative changes in fibrinogen, i.e., increased or decreased reactivity, have not been observed in this laboratory. In a series of coagulation studies,[19-23] Fraction I solution with optimum fibrinogen concentration[19] has been employed as the reagent for the thrombin titration. This reagent has the same reaction time with one unit of thrombin, whether prepared from fresh or stored plasma.

SUMMARY

Normal human plasma stored at refrigerator temperature up to seven and a half months, frozen at –25° C. for fifteen months, and in a dried form from four to six years was analyzed for fibrinogen by two procedures. Results indicated that fibrinogen is stable in plasma stored under these conditions.

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LABORATORY METHODS

A NEW METHOD FOR THE DETERMINATION OF FIBRINOGEN IN SMALL SAMPLES OF PLASMA

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THE techniques currently available for measuring the amount of fibrinogen in small samples of plasma are tedious and time consuming. Recently, Morrison[1] reviewed the methods which have been used for this determination. In one approach, fibrinogen is precipitated from plasma by adding various salts in high concentration. However, fibrinogen cannot be separated satisfactorily in this way from other plasma globulins with similar solubilities. A second approach is to convert fibrinogen to fibrin, and then to measure the amount of fibrin chemically or physically. The accuracy of methods utilizing this principle depends upon the completeness with which the fibrin can be washed free of other proteins. This washing process is difficult because fibrin cannot be separated from diluted serum by centrifugation at ordinary speeds, using techniques previously published.

The present report describes a simplified method for the determination of fibrinogen in plasma. Plasma is pipetted into a centrifuge tube containing crushed glass and a dilute solution of thrombin. Sufficient thrombin is used to shorten the time necessary for complete clotting three- to sixfold compared with older methods. The mixture is agitated while clotting occurs. The fibrin strands as they form adhere to the particles of glass. The fibrin can then be separated from the serum by centrifugation at low speeds. The amount of fibrin thus precipitated is determined chemically. The technique to be described permits the accurate and rapid determination of the amount of fibrinogen in samples of normal plasma as small as 0.1 ml., one tenth the amount used in methods previously described.

METHODS

Nomenclature.—The term *fibrinogen* will be used to describe the component of plasma which is clotted by thrombin. The term *fibrin* will refer to this clotted fibrinogen, as measured by the technique described. Since other plasma proteins are probably adsorbed onto this clot, the term *fibrin* will not be used to imply a purified protein. Alkaline solutions of fibrin react with Folin and Ciocalteu phenol reagent. This property of fibrin will be referred to as its *tyrosine-like activity.*

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Procedure for the Determination of Fibrinogen in Plasma.—Venous blood was drawn from the antecubital vein of human subjects. The blood was immediately transferred to Pyrex centrifuge tubes containing 0.2 ml. of an oxalate mixture for each 5 ml. of blood. The oxalate mixture consisted of 0.8 mg. of potassium oxalate and 1.2 Gm. of ammonium oxalate in each 100 ml. of solution.[2] In a few instances citrated blood was used instead. The plasma was separated by centrifugation for ten minutes at 2,000 r.p.m. in a Sorval angle centrifuge (rim diameter 10 in.).

Pyrex glass was crushed in a mortar so that the diameter of the largest particles was approximately 0.5 millimeter. No attempt was made to grade this *crushed glass,* nor to remove any glass dust. The crushed glass was used repeatedly, and washed between each use with chromic acid.

To a 40 ml. round-bottomed Pyrex centrifuge tube were added, in succession, approximately 0.5 ml. of crushed glass, 10 ml. of 0.85 per cent *sodium chloride solution,* and 0.05 ml. of a solution of *thrombin* (Parke Davis) containing 1,000 National Institutes of Health units per milliliter. Finally, 0.5 ml. of the plasma to be analyzed were pipetted into the tube, which was then agitated with an oscillatory motion. When 0.5 ml. of plasma were not available, samples of plasma as small as 0.1 ml. were used. As clotting occurred, the fibrin strands adhered to the particles of glass. With active preparations of thrombin, clotting appeared to be complete within two to three minutes. Sometimes clotting continued after shaking had been discontinued. Further agitation and rotation of the tube resulted in adhesion of this new-formed clot to the fibrin already attached to the glass.

After approximately ten minutes, the tube containing clotted fibrin was centrifuged for five minutes at 2,000 r.p.m. in the angle centrifuge. The supernatant solution was decanted and discarded. Next, 10 ml. of sodium chloride solution were added to the fibrin clot. The clot was carefully expressed against the wall of the centrifuge tube with a glass rod. The fibrin was again separated by centrifugation for three minutes, washed once more with 10 m. of saline solution, and recentrifuged for three minutes. The supernatant solution was discarded after each centrifugation.

The amount of fibrin adherent to the crushed glass was determined by a minor modification of the method described by Wu[3] and others.[4, 5, 6] One milliliter of 10 per cent (w/v) *sodium hydroxide solution* was added to the centrifuge tube containing crushed glass and fibrin. The tube was then heated in a boiling water bath for ten minutes.[7] Evaporation was minimized by keeping the bath covered during this time. After the tube cooled, 7 ml. of water were added, followed by 3 ml. of 20 per cent (w/v) *sodium carbonate solution* and 1 ml. of Folin and Ciocalteu *phenol reagent* (Hartmann Leddon Co.).[8] A blue color resulted which was fully developed within ten minutes. This period of time was adequate in the presence of crushed gass, whereas in its absence color development required thirty minutes. A 1 ml. aliquot of colored solution was diluted with 3 ml. of water. A blank was prepared by exactly duplicating the procedure outlined, adding thrombin to glass, centrifuging and washing the glass, and so forth, except for the addition of plasma. The intensity of the color was read against the blank in a Coleman Junior spectrophotometer, at a wave length of 650 mμ, using cuvettes with an internal diameter of 10 millimeters.

The amount of fibrin in the plasma tested was estimated by comparing the intensity of the developed color with that of 1 ml. of a standard solution of *tyrosine.* This standard contained 200 mg. of tyrosine per liter of 0.1N hydrochloric acid solution. One milliliter of tyrosine solution was pipetted into a 40 ml. centrifuge tube. In succession, 1 ml. of sodium hydroxide solution, 6 ml. of water, 3 ml. of sodium carbonate solution, and 1 ml. of phenol reagent were added. A blue color resulted which was similar to that found in the reaction of fibrin and phenol reagent. One milliliter of the colored solution was diluted with 3 ml. of water and after ten minutes the intensity of the blue color was read against a blank. This blank was prepared in the same way as the standard solution, except that 1 ml. of water was substituted for the tyrosine. It will be noted that this blank is not identical with that used for reading the color of solutions of fibrin.

The optical density of the developed color was directly proportional to the concentration of fibrinogen or tyrosine until an amount equivalent to 0.35 mg. of tyrosine was reached. When the intensity of color developed in an unknown solution was greater than that produced by 0.35 mg. of tyrosine, the equivalent amount of tyrosine was estimated with the aid of a calibration curve.

The data for developing the calibration curve were as follows. The optical density of the color developed in the reaction between solutions of fibrinogen or tyrosine and Folin and Ciocalteu reagent is shown in Fig. 1. Varying amounts of tyrosine were diluted with water to 7 milliliters. Then, 1 ml. of 10 per cent sodium hydroxide solution, 3 ml. of 20 per cent sodium carbonate solution, and 1 ml. of phenol reagent were added in succession. After ten minutes, 1 ml. of this colored solution was diluted with 3 ml. of water and the intensity of the color was read against a blank. The optical densities of the colors developed in solutions of 0.02 to 0.3 mg. of tyrosine are plotted with cross marks (+).

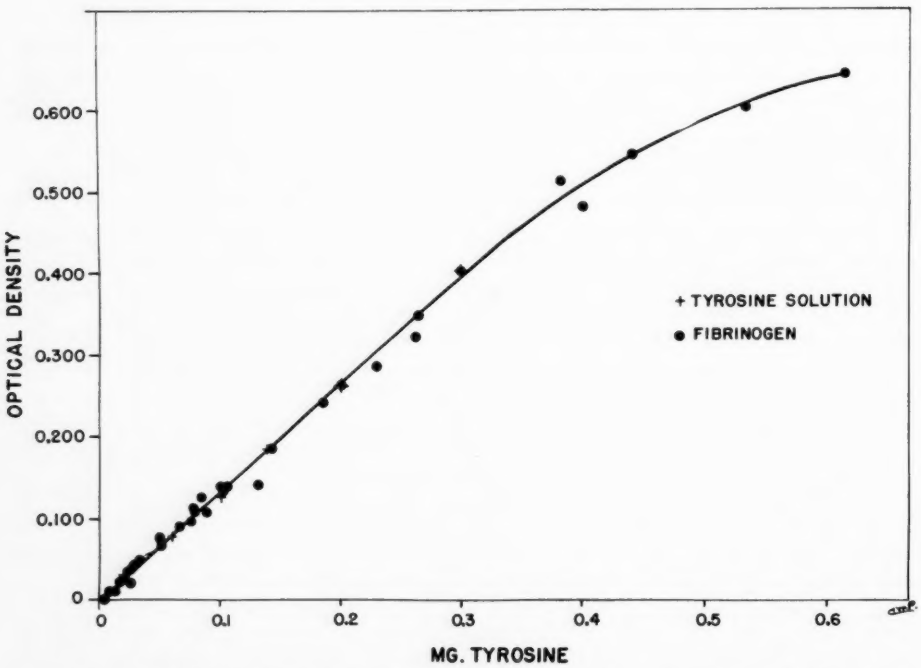

Fig. 1.—Calibration curve for determining the relationship between optical density and concentration of fibrinogen.

A sample of oxalated plasma was diluted to varying degrees with sodium chloride solution. The tyrosine-like activity of the fibrinogen in 0.5 ml. of each dilution of plasma was determined by the technique described in this section. The tyrosine-like activity of one such dilution was plotted arbitrarily on the tyrosine curve. The activity of the other dilutions was then plotted relative to this first point, in proportion to the degree of dilution. For example, the optical density of the fibrinogen of a sample of 0.5 ml. of plasma was 0.262. This point was plotted directly on the tyrosine curve at a point equivalent to 0.2 mg. of tyrosine. The optical density of the fibrinogen of 1.0 ml. of plasma was then plotted on the curve at a point equivalent to 0.4 mg. of tyrosine. The data of many such experiments were then plotted in the same manner. In this way it was possible to determine the relationship between optical density and the concentration of fibrinogen beyond the point where optical density was directly proportional to its tyrosine-like activity.

The amount of fibrinogen in the sample of plasma was calculated by multiplying the weight of tyrosine giving an equivalent amount of color by the factor 11.7. This conversion factor, which differs from that described by others,[5-7] was determined by measuring the tyrosine-like activity and the nitrogen content of duplicate samples of fibrin. The total nitrogen content of the fibrin was determined by the method of Koch and McMeekin,[9] and the weight of the fibrin was then obtained by multiplying its nitrogen content by 5.91.[10] In four experiments, the weight of the fibrin was, on the average, 11.7 times the weight of an equivalent amount of tyrosine; the standard error of the mean was ±0.3. The accuracy of this conversion factor was checked by measuring the amount of fibrinogen in a large sample of plasma gravimetrically. In two such experiments, the conversion factor was 12.4 and 11.6. The average value determined gravimetrically did not differ significantly from that obtained by the Kjeldahl method. When the amount of fibrinogen in 100 ml. of plasma was reported, the calculated answer was multiplied by 1.08 to correct for the dilution of plasma by the anticoagulant solution.

Determination of the amount of fibrinogen in plasma was usually made in duplicate. With samples of plasma as small as 0.1 ml., the difference between the two values obtained was usually less than 10 per cent of the mean value and was often considerably less. In recovery experiments, the fibrinogen contents of mixtures of plasma and purified fibrinogen,* determined chemically, varied from 99.5 to 110.0 per cent of that calculated from the sum of the fibrinogen contents of its components. These data suggest that a small error may be introduced into the determination of plasma fibrinogen by the absorption of other proteins on the fibrin clot.

RESULTS

The Concentration of Fibrinogen in Normal Plasma.—The concentration of fibrinogen in plasma was determined in nineteen normal men and fourteen normal women. In the men, the fibrinogen content of plasma varied from 193 to 415 mg., averaging 272 mg. per 100 milliliters. The standard deviation of this mean was 60.1, and the standard error, 13.8 mg. per 100 milliliters. In the women, the fibrinogen concentration of plasma varied from 164 to 366 mg. per 100 milliliters. The mean value of the fibrinogen concentration in the female subjects was 294 mg. per 100 ml., with a standard deviation of 60.3 and a standard error of 16.1. In other words, there was no significant difference between the concentration of fibrinogen in the plasma of men and women. The values obtained coincided with those reported by Gram.[11]

SUMMARY

In determining the fibrinogen content of plasma, a major difficulty is its separation from other components of the plasma. A method has been described in which plasma was clotted by thrombin, in the presence of crushed glass. Sufficient thrombin was used to shorten the time necessary for complete clotting to occur. The fibrin strands adhered to the crushed glass and could be separated and washed by centrifugation. This technique permitted more rapid and accurate separation of fibrin from plasma than was possible with previously described methods, and required one-tenth the amount of plasma. The amount of fibrin adherent to the crushed glass was determined by measuring its tyrosine-like activity by modifications of the method of Wu which materially shortened the time necessary for the procedure. The complete determination could

*Purified bovine fibrinogen was obtained through the courtesy of Dr. Walter Seegers.

be performed in about one hour, or one half the time required by methods previously reported. Moreover, the technique lends itself to multiple simultaneous determinations more readily than the older methods. With the technique described, the concentration of fibrinogen in plasma averaged 272 mg. per 100 ml. in adult men and 294 mg. per 100 ml. in adult women.

Dr. Charles P. Winsor and Dr. Clifford A. Bachrach of the Department of Biostatistics, School of Hygiene and Public Health, The Johns Hopkins University, aided in the statistical interpretation of the data presented.

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A COLORIMETRIC METHOD FOR THE DETERMINATION OF DICUMAROL (3,3′-METHYLENEBIS (4-HYDROXYCOUMARIN)) IN PLASMA

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ALTHOUGH the biologic activity of Dicumarol, 3,3′-methylenebis (4-hydroxycoumarin), has long been measured by its effect on prothrombin time in man and animals (e.g. the procedure of Link and co-workers[1]), only recently have there been reports of chemical methods for its determination.

In a previous investigation carried out in the laboratories of Professor Karl Paul Link, a method was devised for the determination of Dicumarol in biologic materials[2] and this procedure was applied to whole blood.[3] The method for the determination of Dicumarol is, briefly: (a) extraction of the Dicumarol from the acidified tissue with benzene, (b) reextraction of the benzene phase with alkali, and (c) determination of the ultraviolet absorption at 313 mμ of the alkaline extract containing the Dicumarol. In the presence of interfering substances, chromatographic adsorption was performed between steps (a) and (b).

A similar spectrophotometric procedure for the determination of Dicumarol in biologic fluids has recently been reported by Axelrod and co-workers.[4]

For clinical and physiologic investigations, a simpler method for the estimation of Dicumarol is desirable. Such a method has been devised by the combination of the foregoing extraction procedure with a colorimetric method involving the use of the Folin-Ciocalteu phenol reagent.[5]

METHODS

Reagents.—1. Dicumarol standard solution: 100.0 mg. of Dicumarol (Abbott) are dissolved in 200 ml. of water containing 3 ml. of 5 per cent sodium hydroxide. The solution is then diluted to 1 liter and stored in the refrigerator. It is stable for a least one week.

2. 1.5N sulfuric acid.

3. Glycine buffer: 7.0 Gm. of glycine are dissolved in 1 liter of water and the solution is then adjusted to pH 9.7 with 5 per cent sodium hydroxide. The buffer is stored in the refrigerator, and a small amount of toluene is added to prevent bacterial contamination. If the buffer is to be used for ultraviolet spectrophotometric analysis, the toluene must be omitted.

4. Folin-Ciocalteu phenol reagent: The commercial reagent (Central Scientific Company) is stored in a brown bottle in the refrigerator.

5. Saturated sodium carbonate solution.

Standard Curve.—Dilutions of the stock solution of Dicumarol are made to the concentration range 5 γ to 50 γ per milliliter.

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To standard Evelyn colorimeter tubes (Rubicon Company), solutions are added in the following order: 1.0 ml. of standard Dicumarol solution (1.0 ml. of water for the blank), 4.0 ml. of glycine buffer, 1.0 ml. of water, 1.0 ml. of phenol reagent (shake), 3.0 ml. of sodium carbonate (shake). The blank should be colorless. Thirty minutes after addition of the sodium carbonate the optical density is determined in the Evelyn colorimeter (660 mμ filter). The relationship of optical density to concentration (Fig. 1) although not linear is reproducible.

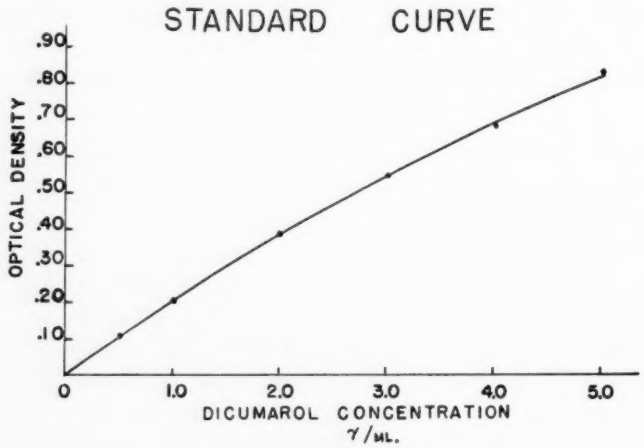

Fig. 1.

Determination of Dicumarol in Plasma.—To a glass-stoppered 30 ml. centrifuge bottle (Maizel-Gerson reaction vessel, Wilkens-Anderson Company), are added 4 ml. of plasma (or serum), 15 ml. of benzene, and 0.25 ml. of 1.5N sulfuric acid, and the contents are immediately mixed. After all tubes are prepared and stoppered, they are shaken vigorously in a mechanical shaker for not less than thirty minutes.*

The tubes are centrifuged for five minutes, a 10.0 ml. aliquot of the benzene layer is transferred to a second bottle containing 5 ml. of the glycine buffer, and the mixture is shaken vigorously for three minutes. It is then centrifuged for three minutes, and the benzene layer is carefully removed with a water aspirator. To a 4.0 ml. aliquot of the remaining glycine layer placed in a colorimeter tube, solutions are added in the following order: 2.0 ml. of water, 1.0 ml. of phenol reagent (shake), 3.0 ml. of sodium carbonate (shake). The blank contains 4.0 ml. of buffer plus the water, phenol reagent, and sodium carbonate. The standard contains 1.0 ml. of Dicumarol solution in place of 1.0 ml. of water. The optical density is measured thirty minutes after the addition of the sodium carbonate.

The blood of patients receiving Dicumarol frequently contains such a high level of the compound that less than a 4.0 ml. aliquot of the final buffer extract must be used. In such cases the volume must be adjusted to 4.0 ml. with glycine buffer.

RESULTS

Normal Plasma.—Using the method outlined, the values shown in Table I were obtained for a number of normal plasmas. The mean of these values was equivalent to 0.03 mg. per cent of Dicumarol, with a standard deviation of 0.008 mg. per cent. In subsequent studies on plasmas of patients receiving Dicumarol,

*A simple holder for the tubes may be prepared by boring holes of the correct diameter in two pieces of wood. The wood is not drilled completely through, so that one piece serves as a rack, while the other serves as a cover. The cover is secured to the rack by bolts and wing nuts. Holders can be made in this manner which can handle eight or more tubes with no danger of breakage or loss of material.

TABLE I. RECOVERY OF DICUMAROL ADDED TO PLASMA

PLASMA ALONE (MG. %)	PER CENT DICUMAROL RECOVERED*	
	10 γ ADDED TO 4 ML. PLASMA	30 γ ADDED TO 4 ML. PLASMA
0.025	98	95
0.025	100	94
0.042		95
0.027	105	98
0.013	96	94
0.030	94	96
0.018	101	99
0.040	94	95
0.042		
0.040	94	95
0.023	105	99
0.03 ± 0.008†	98.5 ± 4.5†	96.0 ± 2.1†

*These values have been corrected for the normal values shown in the column Plasma Alone.

†Mean ± S.D.

this mean value for normal plasma was subtracted from the total Dicumarol found.

Recoveries of Dicumarol.—When Dicumarol was added to normal plasma, the recoveries indicated in Table I were obtained. The mean recovery was 98.2 per cent ± 4.5 per cent at the 10 γ level, and 96.2 per cent ± 2.1 per cent at the 30γ level.

Dicumarol Blood Levels of Patients Treated With Dicumarol.—In six patients receiving various therapeutic dosages of the drug, the Dicumarol content of the blood was found to range from 1.3 to 3.7 mg. per cent.*

Comparison of the Colorimetric and Ultraviolet Spectrophotometric Results.—Aliquots of the glycine extracts from plasma of patients receiving Dicumarol prepared as previously described were washed twice with ether to remove traces of benzene. The absorption spectra of such extracts were typical of Dicumarol, with maxima at 313 millimicrons. Quantitative measurements at 313 mμ showed excellent agreement with those made by the colorimetric method (Table II).

TABLE II. PLASMA CONCENTRATION OF DICUMAROL IN TREATED PATIENTS

PATIENT	COLORIMETRIC METHOD (MG. %)	SPECTROPHOTOMETRIC METHOD (MG. %)
1	1.34	1.32
2	3.78	3.74
3	2.06	2.05
4	3.39	3.18
5	1.97	1.80

Interfering Drugs.—A number of common drugs were carried through the extraction procedure in high concentrations, to determine whether they would give color with the phenol reagent. Luminal, digitoxin, and heparin gave no

*A more complete study of the relationship of blood level to dosage and prothrombin time is now being undertaken by E. B. Bay and M. Seide.

color. Penicillin and morphine yielded slight color. Aureomycin, vitamin K, and phenol produced considerable color. It has not been determined whether these substances interfere at levels found in plasma. Salicylate at concentrations comparable to therapeutic plasma levels yields considerable color with this reagent.

SUMMARY

A sensitive colorimetric method for the determination of Dicumarol in plasma has been described. The procedure is simple and easily adaptable to routine use.

Recoveries of Dicumarol added to plasma at two concentration levels averaged 96 and 98 per cent.

Normal plasma gave a value equivalent to 0.03 mg. per cent of Dicumarol. This figure is small when compared with the plasma values (1.3 to 3.4 mg. per cent) obtained from patients being treated with Dicumarol at various therapeutic dosage levels. However, for highest accuracy (e.g., where the Dicumarol plasma concentration is less than 1.0 mg. per cent) a correction may be made by subtracting the mean normal value of 0.03 mg. per cent.

When utilizing this procedure, it must be borne in mind that certain other medications may produce a high false value for Dicumarol. This is true for phenolic compounds (e.g., salicylates) and possibly vitamin K.

The authors are indebted to Dr. Albert Dorfman and Dr. Karl Paul Link for their valuable suggestions, and to Dr. E. B. Bay for supplying the necessary blood samples.

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A CULTURE FLASK FOR THE ESTIMATION OF THE GROWTH OF MICROORGANISMS BY OPTICAL METHODS

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THE factors which determine the effectiveness of aeration on the growth of microorganisms have been investigated in several laboratories.[1, 2, 3] In every instance, the procedures used for the estimation of growth either required the removal of samples at intervals or depended upon a single determination after a standard incubation period. The problem of determining the length of the lag period and the growth rate during the exponential growth phase has led to the development of a specially designed culture flask. With this flask, periodic measurements of the light absorbed by the cell suspension and medium can be made directly on the entire culture with the Evelyn photoelectric colorimeter.

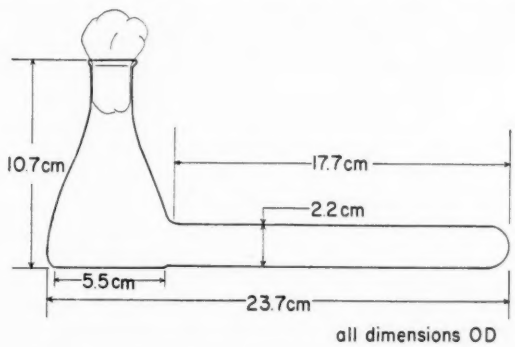

Fig. 1.—Culture flask for the turbidimetric estimation of the growth of microorganisms.

The culture flask (Fig. 1) is similar in principle to the one designed by Henry and Henry,[4] but its construction is modified to provide a greater surface for aeration. By shaking the flasks in a Warburg bath, aeration is provided at rates which are nonlimiting for growth. Absorption tubes, used with the Evelyn photoelectric colorimeter which had been selected for optical uniformity, were sealed to 125 ml. Erlenmeyer flasks blown from Kimble's standard flint glass stock (S/F).*

The flasks are clamped to the shaking rack of a Warburg constant-temperature bath with the absorption tube in a horizontal plane, perpendicular to the direction of shaking. It is important to mount the flasks parallel to one another in order to assure the same degree of aeration in each. In Warburg baths available commercially, there is room for at least six culture flasks to be shaken simultaneously.

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*These flasks were made by the L. G. Nestor Company, Millville, N. J.

Preliminary experiments on the growth of *Torulopsis utilis* (ATCC #8206) and *Saccharomyces cerevisiae* (#15.56 obtained through the courtesy of Dr. A. S. Schultz) at 30° C. indicate that the growth rate is independent of the aeration when the flasks are shaken at 100 cycles per minute. The yeasts were grown in these experiments on a medium devised by Atkin and co-workers.[5]

SUMMARY

A culture flask is described which permits the growth of microorganisms to be followed by optical density determinations. The small size of the flask allows multiple determinations under nearly identical conditions of aeration.

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ELECTRICALLY OPERATED BUNSEN-TYPE BURNER*

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INTRODUCTION

THE need for an electrically operated Bunsen-type burner is obvious. Many laboratories install gas at a great expense for the specific purpose of operating burners. Often laboratory procedures are discouraged because of the lack of gas. The burner described herein can perform practically all of the functions of the gas-operated type but has the advantages of flexibility, ease of operation, time saving, and reduction of hazards to the operator.

The electrically operated burner is a simplified modification of a previous burner described by me.‡

DESCRIPTION OF INSTRUMENT

(See Figs. 1, 2, 3, and 4 of the burner from various angles.) The heating unit is composed of a lava cylinder 3¼ in. long with an outside diameter of ⅞ in. and a central cavity ½ in. in diameter which runs the entire length of the core. Heater wire of 28 gauge is wound around the threaded column submerging the wire from the surface. The heater is operated by a toggle switch in the base. A movable deflector can be rotated 360 degrees and functions to concentrate the heat radiation away from the operator. Slots in the base are provided for needle holders.

METHOD OF OPERATION

Bacteriologic Procedures.—For bacteriologic procedures the deflector is rotated toward the operator and in the upper quadrant. (Fig. 1.) The heater is turned on and when the lava within the cylinder glows the instrument is ready for use. The bacteriologic loop or needle is placed in the cavity of the core and allowed to rest there without holding, being certain that the tip of the loop does not extend beyond the glowing portion of the lava. If longer needles are preferred, they may be passed slowly through the cavity as through a flame. Contact of the loop with the glowing lava gives the best results.

By incinerating within a cavity, spattering is confined within it, thus reducing the hazards of working with virulent organisms. If test tubes are used, the mouths are sterilized either by holding them directly against the outside of the core or by rolling the end of the tube over the core. Transferring proceeds in the usual manner. Touching cotton plugs to the coils will ignite them. The burner may be grasped by the base (Fig. 2) and used to sterilize the field of operation, transferring into Petri dishes, and so on.

Clinical Laboratory Procedures.—For heating fluids in test tubes, move the deflector away from the operator to the lower quadrant. Any portion of the column of fluid may be heated by simply rolling the tube over that portion. (Fig. 3.) The gradual elevation of temperature minimizes bubbling over and sputtering.

Heating of fluids in beakers, etc., is accomplished by using a special type of stand. (Fig. 4.) This permits the bottom of the container to rest on the stand and just clear the core. (Not more than ⅛ in. clearance, but should not touch core.)

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*Manufactured by Precision Scientific Company, 3737 West Cortland Ave., Chicago, Ill.

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‡Rosenthal, Sol Roy: Electrically operated burner and bacterial loop, J.A.M.A. **108**: 1969, 1937.

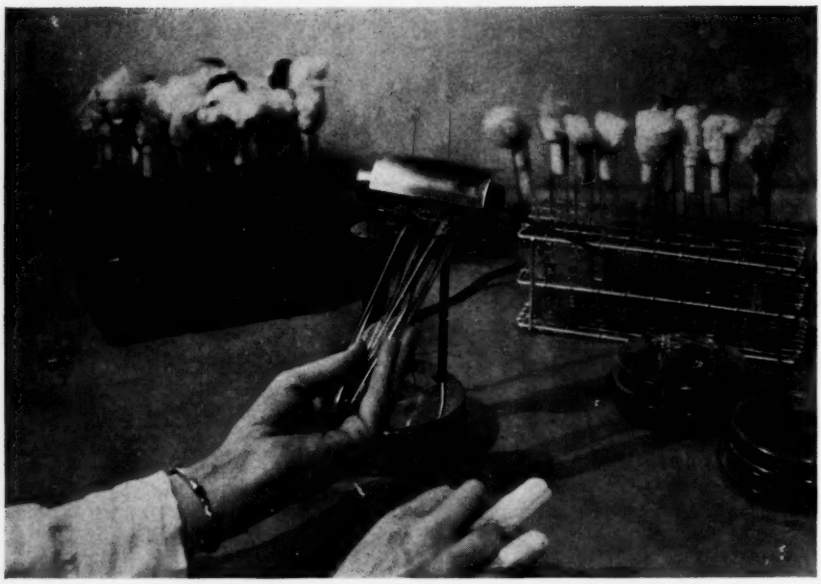

Fig. 1.—For bacteriology. Inoculation of cultures: The inoculating needle or loop rests in the cavity of the core and is always ready for use. The mouths of the test tubes are held directly on the coils or may be rolled over the coils. Note that position of shield is facing the operator.

For long needles or loops, hold the handle so that the wire touches the glowing inside wall of the core until it glows itself, then slowly pass back and forth for sterilization.

Fig. 2.—Mobility of Bacti-burner. The burner may be grasped at the base to use for sterilizing the field of operation, transferring into Petri dishes, in water and milk bacteriology, in bedside blood cultures, and so on.

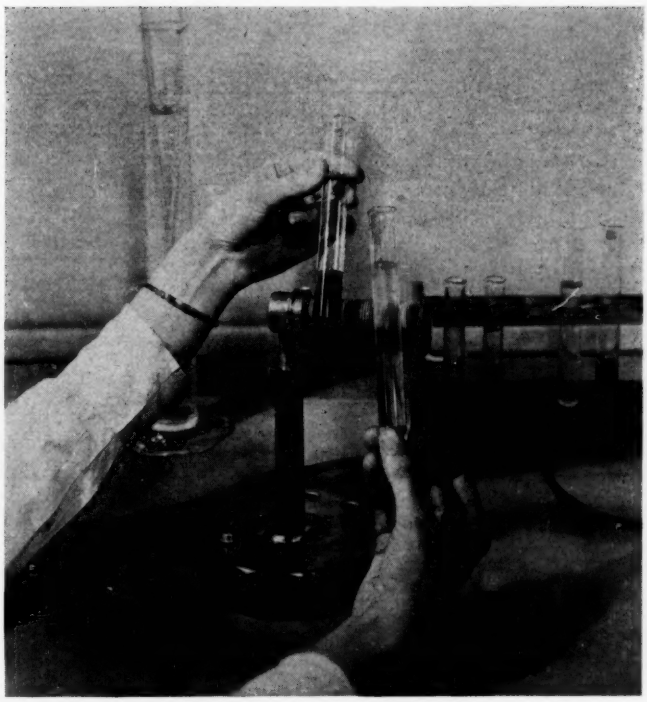

Fig. 3.—For urine analysis. Heating fluids in test tubes: Roll the test tube directly over the glowing coils so that glass touches the ceramic portion. Any portion of the fluid column may be heated. Note that the shield is away from the operator.

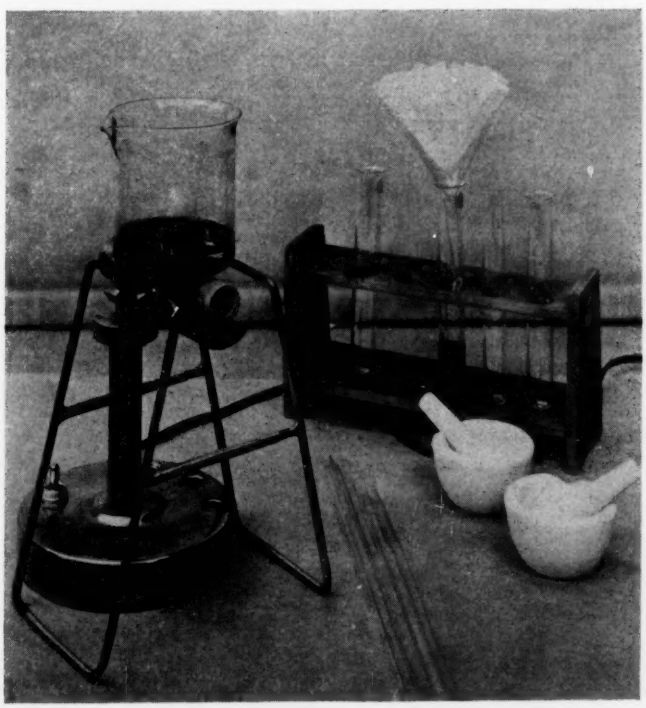

Fig. 4.—For chemistry. Heating of fluids in larger containers: Use special stand as illustrated for this purpose. The container rests on the stand and just clears (⅛ in.) the coils. The special stand is adjustable horizontally by means of moving the lateral sides in or out.

DISCUSSION AND SUMMARY

This electrically operated burner makes it possible to do clinical laboratory work in any room where there is an electrical outlet. For the bacteriologist its "spatter-proof" feature is important, since it minimizes the dissemination of virulent organisms during bacteriologic procedures. As one or more loops may be left in the cavity of the core without the necessity of holding them, both time and labor are economized. Some experiments with sizes of holders and types of loop employed should be done so that the procedure may become automatic. A metal holder ⅛ in. in diameter and 6 in. long that grasps a 2¾ in. loop of twisted chrome wire has been found ideal for this instrument. The rigidity of this loop makes it possible to rest it in the cavity of the column without bending it, and the narrow gauge of the holder makes it possible to have several loops in the cavity at one time. A narrow collar of rubber tubing is recommended to cover the holder where it is to be held, since metal holders will heat up if left in the cavity over long periods. However, any convenient loop and holder may be used and the conventional manner of passing through the "flame" or cavity of the core is followed. The loop should scrape the glowing lava for the best results. The horizontal position of the cylinder makes it easier for the operator, compared with the vertical flame of the gas method.

The gradual heating of fluids as accomplished by this heater makes it ideally suited for all clinical laboratory methods where heating is necessary.

The advantages over the gas type of burner are its flexibility of use, the absence of a "cold" portion of the flame, the elimination of failure of the flame due to kinking of the gas tubes or to drafts, the elimination of the use of matches, the silence of operation, and so forth. Currents in the field of operation are reduced, since the pressure of gas is 6 in. and makes for circulation of air, which introduces the possibility of contamination.